Identification and Characterization of an Amphipathic Leucine 
Zipper-like Motif in Escherichia coli Toxin Hemolysin E 

PLAUSIBLE ROLE IN THE ASSEMBLY AND MEMBRANE DESTABILIZATION*

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Hemolysin E (HlyE) is a 34 kDa protein toxin, recently isolated from a pathogenic strain of Escherichia coli, which is believed to exert its toxic activity via formation of pores in the target cell membrane. With the goal of understanding the involvement of different segments of hemolysin E in the membrane interaction and assembly of the toxin, a conserved, amphipathic leucine zipper-like motif has been identified. In order to evaluate the possible structural and functional roles of this segment in HlyE, a 30-residue peptide (H-205) corresponding to the leucine zipper motif (amino acid 205–234) and two mutant peptides of the same size were synthesized and labeled by fluorescent probes at their N termini. The results show that the wild-type H-205 binds to both zwitterionic (PC/Chol) and negatively charged (PC/PG/Chol) phospholipid vesicles and also self-assemble therein. Detailed membrane-binding experiments revealed that this synthetic motif (H-205) formed large aggregates and inserted into the bilayer of only negatively charged lipid vesicles but not of zwitterionic membrane. Although both the mutants bound to zwitterionic and negatively charged lipid vesicles, neither of them inserted into the lipid bilayers nor assembled in any of these lipid vesicles. Furthermore, H-205 adopted a significant helical structure in membrane mimetic environments and induced the permeation of monovalent ions and release of entrapped calcein across the phospholipid vesicles more efficiently than the mutant peptides. The results presented here indicate that this H-205 (amino acid 205–234) segment may be an important structural element in hemolysin E, which could play a significant role in the binding and assembly of the toxin in the target cell membrane and its destabilization.

Proteinaceous toxins are often major weapons of pathogenic organisms to invade their target cells and overcome the host defense system. Hemolysin E, also known as cytolytic A (ClyA) and silent hemolysin A (SheA), is a recently identified cytotoxic agent from a pathogenic strain of Escherichia coli (1, 2, 3). This 34 kDa protein toxin is believed to be involved in the intra- and extra-intestinal infection caused by the pathogenic strain of E. coli (3). Hemolysin E is expressed during anaerobic growth of E. coli in mammalian intestine (3, 4). It is interesting to mention that hemolysin E gene although found in non-pathogenic strains of E. coli, it remains silent under laboratory conditions. However, it is positively regulated by several regulatory proteins, which include SlyA and MprA (1, 3, 5–8). This probably suggests that hemolysin E is expressed in response to appropriate environmental signals.

Bacteria expressing hemolysin E are able to lyse erythrocytes from several mammalian species including human in both solid and liquid media (1, 2). Macrophages grown in tissue culture media are also lysed by an E. coli strain expressing HlyE (9). Sequence comparisons show that typhoid-causing bacterium Salmonella typhi and dysentery-causing organism Shigella flexneri express highly homologous proteins to HlyE of E. coli (10). It has been shown that the expression of HlyE in the absence of the RTX-family toxins is sufficient to give a hemolytic phenotype of E. coli (11). Recently hlye homologous genes have been cloned from human-specific Salmonella enterica serovars Typhi and Paratyphi A and the hemolytic activity of the expressed proteins have been characterized in detail (12). All these data indicate that HlyE similar to other hemolysins (13, 14) is likely to be a significant virulence factor responsible for pathogenic infection in human. Experiments in lipid bilayer indicated that hemolysin E forms pores in membrane (4, 7). This pore-forming activity of the toxin is considered to be associated with the infection of the pathogenic organism in the target cell.

Crystal structure of the water-soluble form of the toxin has been solved at high resolution, which showed that the toxin mainly consists of long helical structures (10). In a recent structure-function study, it has been reported that the deletion of 37 amino acids, by site-directed mutagenesis, from the C-terminal rendered the toxin non-hemolytic (15). However, this loss of hemolytic activity of hemolysin E may be due to misfolding of the protein, resulted from the deletion of amino acids (15).

Although hemolysin E has been characterized biochemically and biophysically by several groups, a number of questions remain to be answered. For example, it is not known how this toxin assembles in membrane and which part of the toxin is involved in membrane interaction and pore formation.

With the primary objective to find out possible membrane-interacting domains in hemolysin E, a conserved, amphipathic leucine zipper-like motif has been identified. This leucine zipper-like motif (amino acid 205–234) contains three heptads with two heptadic positions occupied by leucine and isoleucine and other two positions occupied by valine and phenylalanine. Three peptides including the wild type (H-205) and two mutants have been designed and synthesized from this hemolysin E segment. One of the mutants (Mu1-H-205) is a scrambled peptide in which the positions of two heptadic amino acids,
leucine and isoleucine, were interchanged with the positions of the two hydrophilic amino acids lysine and serine keeping the amino acid composition of the peptide the same as the wild type. In the other mutant (Mu2-H-205), a heptad leucine was replaced by another hydrophobic and helix-forming amino acid alanine. In order to detect its membrane interaction and assemble in aqueous and in membrane environment, peptides were labeled with fluorescent probes NBD (7-nitrobenz-2-oxa-1,3-diazole) and rhodamine (tetramethylrhodamine, Rh). Secondary structures, membrane interaction, assembly and membrane bilayer destabilization ability of H-205 and its analogues were studied. The results obtained from these studies have been discussed in terms of the role of this conserved segment in hemolysin E-mediated binding and destabilization of the target cell membrane.

EXPERIMENTAL PROCEDURES

Materials—Rink amide MBHA resin (loading capacity: 0.4–0.8 mmol/g) and all the N-,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 (DIPEA) were purchased from Sigma. Dichloromethane, N,N'-dimethylformamide (DMF) and piperidine were of standard grades and procured from reputed local companies. Acmotin (HPLC grade) was procured from Merck, India while trifluoroacetic acid, trifluoroethanol (TFE), and sodium dodecyl sulfate (SDS) were purchased from Sigma. Egg phosphatidylcholine; PC, phosphatidylglycerol; PG, cholesterol.

Peptide Synthesis, Fluorescent Labeling, and Purification—All the peptides were synthesized manually on solid phase. Stepwise solid phase syntheses were carried out on rink amide MBHA resin (0.15 mmol) utilizing the standard Fmoc chemistry, employing DIC/HOBt or TBTU/HOBt/DIPEA coupling procedure (16–18). De-protection of a-amino group and the coupling of amino acids were checked by Kaiser test (19) for primary amines and chloranil test for secondary amine (20). After the synthesis was over, each peptide was cleaved from the resin with the aid of Fmoc de-protection of N,N'-diisopropylethylamine and tetramethylrhodamine succinimidyl ester were procured from Molecular Probes (Eugene, OR). The rest of the reagents were of analytical grade and procured locally; buffers were prepared in milli Q water.

Membrane Interaction of Hemolysin E Segment

Membrane Binding Experiments—The affinities of the peptides for phospholipid vesicles were determined by binding experiments as reported earlier (28–30). In brief, 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 set at 467 nm and emission at 531.5 nm. The excitation and emission slits were fixed at 8 and 6 nm, respectively. The contributions of lipid to any of the recorded signal were measured by titrating the unlabeled 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 Equation 2,

$$X_n^* = K_n^* \cdot C_n$$

where $X_n^*$ 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 Beschiaschvili and Seelig (29). $K_n^*$ represents the partition coefficient and $C_n$ indicates the concentration of the free peptide at equilibrium. $X_n^*$ was calculated by extrapolating the fluorescence signal $F_{\text{fluorescence}}$ (fluorescence signal when all the peptides were bound to lipid) from a double-reciprocal plot of $F$ (peptide fluorescence in the presence of lipid) against $[\text{lipid}]$ (lipid concentration). Fraction of peptide bound ($f_b$) was determined by Equation 3,

$$f_b = (F - F_0)/(F_{\text{fluorescence}} - F_0)$$

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 unbond state. If $f_b$ is known, $C_n$ can easily be calculated for each concentration of the lipid. $K_n^*$ can easily be determined from the slope of the plot of $X_n^*$ and $C_n$.

Enzymatic Cleavage Experiments—In order to detect the location of a NBD-labeled peptide in its membrane-bound state, enzymatic cleavage experiments were performed as reported earlier (31, 22). In brief, lipid vesicles made of either PC/Chol or PC/PG/Chol were first added to the NBD-labeled peptide. When major portion of the peptide was bound to the lipid vesicles as detected by the saturation of the fluorescence level, proteinase-k (final concentration, 10.0 μg/ml) was added. In this experiment fluorescence of NBD-labeled peptide was recorded at 525 nm with respect to time (in s) with excitation wavelength set at 467 nm. In the control experiment proteinase-k was first added to NBD-labeled peptide, and then lipid vesicles were added.

Fluorescence Resonance Energy Transfer Experiment—Fluorescence energy transfer experiments were performed with excitation wavelength set at 467 nm and emission range of 500–600 nm. Desired amount of the NBD-labeled peptide was taken in a fluorimeter cuvette. The efficiency of energy transfer ($\varepsilon$) was determined by the decrease in donor fluorescence in the presence of the acceptor as re-

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1 The abbreviations used are: NBD, 7-nitrobenz-2-oxa-1,3-diazole; Fmoc, N-(9-fluorenylethoxycarbonyl); PBS, phosphate-buffered saline; TFE, trifluoroethanol; SUV, small unilamellar vesicles; PC, phosphatidylcholine; PG, phosphatidylglycerol; Chol, cholesterol.
Membrane Interaction of Hemolysin E Segment

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ported earlier (32, 22). The percentage of energy transfer was calculated by Equation 4,

$$E = \left( \frac{I_{00} - I_{0}I_{0}/I_{00}}{I_{00}} \right) \times 100$$

(Eq. 4)

where $I_{00}$ and $I_{0}$ are the fluorescence intensities of the NBD-labeled donor peptide in the absence and presence of the Rho-labeled acceptor peptide respectively at the emission maxima of the donor after correcting the light scattering of the lipid vesicles and emission of the acceptor.

**Membrane Permeability Assays: Dissipation of Diffusion Potential from the Small Unilamellar Lipid Vesicles—**The ability of the peptides to destabilize the phospholipid bilayer was detected by their efficacy to dissipate the diffusion potential across the membrane. The experiments were performed as follows (33, 34). Lipid vesicles were prepared in K+ buffer (50 mM K2SO4/25 mM HEPES sulfate, pH 6.8). Required amounts of the lipid vesicles were mixed with isotonic K+ -free Na+ buffer (50 mM Na2SO4/25 mM HEPES sulfate, pH 6.8) followed by the addition of the potential sensitive dye diS-C3(5). Addition of valinomycin created a negative potential inside the lipid vesicles by the selective influx of K+ ions from the lipid vesicles. As a result of that a quenching of the fluorescence of the dye occurred. When the dye exhibited a steady fluorescence level, peptides were added. Membrane permeability of the peptide was detected by the increase in fluorescence, which resulted from the dissipation of diffusion potential. Fluorescence was monitored at 670 nm with respect to time (s) with excitation wavelength of 620 nm. The peptide-induced dissipation of diffusion potential was measured in terms of percentage of fluorescence recovery ($F_{t}$) as defined by Refs. 21 and 22 in Equation 5,

$$F_{t} = \left( \frac{I_{0} - I_{0}/I_{00}}{I_{00}} \right) \times 100$$

(Eq. 5)

where $I_{0}$ = the observed fluorescence after the addition of a peptide at time t (10 min after the addition of the peptide), $I_{00}$ = the fluorescence recovered after the addition valinomycin and $I_{0}$ = the total fluorescence observed before the addition of valinomycin.

**Calcine Release from Calcine-entraped Lipid Vesicles—**Peptide induced release of calcine from calcine-entraped vesicles is often employed to detect the pore-forming activity of proteins and peptides. Calcine-entraped lipid vesicles were prepared with a self-quenching concentration (60 mM) of the dye in 10 mM HEPES at pH 7.4 as reported earlier (35, 36). Briefly, thin film of lipid (either PC/PG/Chol or PC/Chol) was re-suspended in calcine solution, vortexed for 1–2 min and then sonicated in a bath-sonicator. The non-encapsulated calcine was removed from the liposome suspension by gel filtration using a Sephadex G-50 column. Usually lipid vesicles are diluted to 10-fold after passing through a G-50 column. The eluted calcine-entraped vesicles were diluted further in the same buffer to a final lipid concentration of 3.0 mM for the experiment. Peptide-induced release of calcine from the lipid vesicles was monitored by the increase in fluorescence due to the dilution of the dye from its self-quenched concentration.

Fluorescence was measured at room temperature with excitation and emission wavelengths fixed at 490 and 520 nm, respectively. Calcine release as measured by the fluorescence recovery is defined by the same equation as used to determine the dissipation of diffusion potential. However, in this case $I$, the total fluorescence, was determined after the addition of Triton X-100 (0.1% final concentration) to the dye-entrapped vesicle suspension.

**RESULTS**

Hemolysin E is a recently identified toxin from a pathogenic strain of *E. coli*. Since it is a pore-forming toxin, membrane interaction and assembly therein are the key steps, associated with its mechanism of action. Recently membrane interaction of several protein and peptide toxins like pneumolysin (37), *Vibrio cholerae* cytolsin (38), equinatoxin (39), *E. coli* a-hemolysin (40, 41) *B. thuringiensis* δ-toxin (31, 42) and pardaxin (21) have been studied in detail in order to understand their mechanism of action. Although, pore-formation has been demonstrated by electrophysiological experiments (4, 7) and visualized by electron microscopy (10), membrane-interacting segments of hemolysin E are not yet known. In order to understand the possible contribution of different parts of hemolysin E to its membrane interaction and assembly, we have identified an amphipathic leucine zipper-like motif H-205 (amino acid 205–234) in this toxin. Sequence alignment, shown in panel A of Fig. 1, clearly describes that this particular segment and the heptadic amino acids are conserved in the homologous proteins of the same family. Panel B of Fig. 1 depicts all the sequences of the peptides including their NBD- and Rho-labeled analogues, used in this study. H-205 and two mutants were synthesized by standard solid phase synthesis. Experimental mass of each of the peptides as detected by ES-MS was within 0.5 Da of the respective desired mass. Panel C of Fig. 1 shows the helical-wheel projections of H-205 and Mu1-H-205. Panel D of Fig. 1 represents the Schiffer Edmundson wheel projections of the wild-type H-205, which shows the segregation of hydrophobic and hydrophilic amino acids in opposite sides, indicating amphipathic nature of the peptide.

**Both the Wild Type and Mutant Peptides Derived from the Leucine Zipper-like Motif Bind to the Phospholipid Membrane—**In order to detect the ability of the peptides to bind to phospholipid vesicles, they were labeled by fluorescent probe NBD. 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 (43–45, 30). Fluorescence emission spectra of NBD-labeled H-205, Mu1-H-205, and Mu2-H-205 were recorded in aqueous buffer and in the presence of phospholipid vesicles. The fluorescence spectra of wild-type and mutant peptides exhibited broad emission maxima around 545 nm in phosphate buffer indicating the location of the probe in the hydrophilic environment (46). However, in the presence of zwitterionic PC/Chol (8:1 w/w) and mixture of zwitterionic and negatively charged PC/PG/Chol (4:4:1) phospholipid vesicles fluorescence spectra of the NBD-labeled wild-type and mutant peptides exhibited a blue shift of emission maxima concomitant with significant increase in fluorescence (Fig. 2). These shifts of emission maxima toward shorter wavelength with increased fluorescence indicate the relocation of the probe in the hydrophobic environment due to binding of the peptides to phospholipid vesicles. Wild-type H-205 exhibited an emission maximum of 526–527 nm in the presence of negatively charged PC/PG/Chol lipid vesicles which is much shorter than the emission maximum of 533 nm, characteristic of the location of the NBD probe on surface of the membrane (47). This further suggests that the N-terminal of H-205 might be inserted into the membrane bilayer of the negatively charged lipid vesicles. However, H-205 exhibited an emission maximum of 529–530 nm in only zwitterionic phospholipid vesicles (PC/Chol) indicating the location of its N-terminal more toward the surface of the membrane. NBD-labeled mutant peptides displayed emission maxima within 531–532 nm in the presence of either PC/Chol or PC/PG/Chol lipid vesicles suggesting that their N-terminals were most likely located on the surface of the membrane. These results clearly demonstrate that the synthetic wild-type and mutant peptides derived from this amphipathic leucine zipper-like motif can bind to both negatively charged and only zwitterionic lipid vesicles but their localization is influenced by their amino acid sequence and lipid composition of the membrane.

**Determination of Affinity of the Peptides toward Phospholipid Membrane—**Affinities of H-205 and its analogues to the phospholipid membrane were determined by the binding experiments utilizing their NBD-labeled analogues. Fluorescence signal of the NBD-labeled peptides as a result of their binding to the phospholipid membrane was plotted with respect to lipid/peptide molar ratio. Panel A of Fig. 3 describes four such plots; two each for H-205 and Mu2-H-205 in PC/Chol and PC/PG/Chol lipid vesicles. Since the change in fluorescence of NBD-Mu1-H-205 with change in lipid concentration was very similar to that of NBD-Mu2-H-205, the plots are not shown. All the plots indicate a gradual increase in fluorescence with increase in lipid concentration indicating a progressive binding of
the NBD-labeled peptides to the lipid vesicles. It is interesting to note that NBD-labeled H-205 exhibited a sharp increase in fluorescence in the presence of PC/PG/Chol even at a very low lipid/peptide molar ratio indicating its very high affinity toward the mixture of zwitterionic and negatively charged lipid vesicles. Binding isotherms were generated by plotting $X_b$ with respect to $C_f$ as has been described under "Experimental Procedures." 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-205 exhibited an appreciable affinity for PC/Chol lipid vesicles as indicated (panel C) by its partition coefficient of $1.5 \times 10^4$, which is similar to that of the surface-active peptides, derived from anti-microbial peptides, bacterial toxins and viral fusion proteins (48, 30, 32, 22). The estimated partition coefficient ($1.1 \times 10^4$) of NBD-Mu2-H-205 was slightly lower than the wild type in PC/Chol lipid vesicles. However, NBD-labeled H-205 exhibited significantly high affinity for the mixture of zwitterionic and negatively charged PC/PG/Chol vesicles as determined by the partition coefficient of $2.5 \times 10^5$ (panel B).

The estimated partition coefficient ($2.6 \times 10^4$) of NBD-labeled Mu2-H-205 was lower than the wild-type H-205. These results suggest that all the NBD-labeled peptides derived from the leucine zipper-like motif exhibited appreciable affinity for lipid vesicles except NBD-labeled H-205, which showed remarkably high affinity for negatively charged PC/PG/Chol vesicles. Moreover, the results show that mutation did affect the affinity of the NBD-labeled peptides toward the PC/PG/Chol lipid vesicles. The nature of the binding curve can give an idea about the assembly of a peptide in membrane. The binding isotherms of NBD-labeled H-205 in PC/Chol and the NBD-labeled mutant peptides in PC/Chol or PC/PG/Chol vesicles were linear in nature (panel C), suggesting that the binding of these peptides to membrane was a simple adhesion process and they did not form large aggregates in membrane. However, the binding isotherm of NBD-labeled H-205 in PC/PG/Chol bent downward appreciably and deviated from linearity (panel B). As suggested earlier, this kind of curve indicates the cooperativity in binding of peptides to membrane (48, 30).

NBD-labeled H-205 Appreciably Resists Cleavage by Proteinase-k When It Is Bound to Negatively Charged PC/PG/Chol Lipid Vesicles—In order to determine the location of the peptides onto the membrane, proteolytic cleavage experiments were performed with NBD-labeled peptides in their membrane-bound state (31, 22). The basis of this experiment is that NBD-labeled peptides, bound onto the membrane-surface, are easily cleaved by a proteolytic enzyme like proteinase-k, which can be monitored by the decrease in NBD-fluorescence from the characteristic membrane-bound level. On the other hand, a membrane-inserted peptide will not be accessible to proteinase-k and therefore the NBD-fluorescence will not decrease. Profile a of panel A in Fig. 4 describes the proteolytic cleavage experiment with NBD-labeled wild-type H-205 in PC/PG/Chol lipid vesicles. At time point 1 NBD-labeled H-205 was added to the buffer followed by the addition of PC/PG/Chol lipid vesicles at time point 2. The addition of lipid vesicles resulted in a sharp increase of NBD fluorescence due to binding of the peptide to the lipid vesicles with a fast kinetics. At time point 3 when NBD fluorescence has reached the plateau, indicating a complete binding of the labeled peptide to the membrane, proteinase-k was added. The addition of proteinase-k resulted in a very slow...
decrease in NBD fluorescence indicating that NBD-H-205 appreciably protects itself from the digestion by proteolytic enzyme. The 50% decrease in NBD fluorescence, resulting from the cleavage of NBD-H-205 in its membrane-bound state did not take place even after incubation of 3100 s. This result indicates that at least a part of the peptide was hidden inside the lipid bilayer and hence not cleaved even after a long incubation. A control experiment (profile b) was performed in order to check whether the peptide is cleaved easily or not when it is not bound to membrane. Proteinase-k was added to the NBD-labeled peptide, incubated for sufficient time and then lipid vesicles were added. In this case addition of lipid vesicles resulted only in a small increase of NBD fluorescence (compared with the previous experiment when vesicles were added to NBD-H-205 before proteinase-k treatment) indicating that the peptides were cleaved by the enzyme and therefore unable to bind to the membrane. Similar experiments were carried out with NBD-labeled mutant peptides. For example, a in panel B depicts the experimental profile of NBD-Mu2-H-205. In contrast to the wild-type peptide, NBD-Mu2-H-205 was easily cleaved by proteinase-k in its membrane-bound state as evident from the sharp decrease in NBD fluorescence after the addition of proteinase-k. The time to cause 50% decrease in membrane-bound NBD fluorescence was only ~400 s. The corresponding control experiment (profile b of panel B) suggested that the mutant peptide was also cleaved easily when it was not bound to the membrane. The results of the mutant −1 was similar to that of the mutant-2 and therefore not shown. These results suggest that the wild type and mutant peptides were cleaved easily in aqueous buffer by proteinase-k. However, only the wild-type peptide resisted the proteolytic digestion after binding to PC/PG/Chol lipid vesicles probably due to its insertion into the lipid bilayer. Furthermore, experiments were done with wild type and mutant peptides in the presence of zwitterionic PC/Chol lipid vesicles. It was observed that both wild type and mutant peptides were cleaved promptly when they were bound to zwitterionic lipid vesicles (data not shown).

Wild-type H-205 Adopts Significant Helical Structure in Membrane Mimetic Environments—Circular dichroism studies were employed in order to determine the secondary structure of these peptides in aqueous and membrane mimetic environments. Secondary structures 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. Fig. 5 depicts the circular dichroism studies on these peptides in different environments. All three peptides exhibited mostly random coil structure in PBS and therefore not presented. However, the extent of their helical structure increased significantly in 40% TFE and 1% SDS. The mean residual ellipticity values at 222 nm of the CD spectra of wild-type H-205 in 40% TFE and 1% SDS were 25,356 and 22,607 which correspond to 77.8% and 68.7% helical structure respectively. For Mu1-H-205 mean residue ellipticity values at 222 nm in 40% TFE and 1% SDS were 15,324 and 12,609, which correspond to helicities of 44.4 and 35.6% respectively. The estimated helical structure of Mu2-H-205 in 40% TFE and 1% SDS were 41.8 and 55.9% respectively. The estimated helical structure increased significantly in 40% TFE and 1% SDS. These results indicate that the wild-type H-205 adopted significant helical structure in both 40% TFE and 1% SDS and mutations resulted in the decrease in helical structure.

Only the Wild-type H-205 but Not the Mutants Self-assemble in Phospholipid Vesicles—Proper assembly of hemolysin E is a prerequisite for exhibiting its pore-forming activity. In order to evaluate any possible role of this leucine zipper-like motif in
the assembly of hemolysin E in membrane, fluorescence energy transfer experiments were performed. In this connection it is to be mentioned that the shape of the binding isotherms can detect only large aggregates but not the self-association of a polypeptide to form small sized bundles (22). In order to perform the energy transfer experiments, each of the peptides under investigation, was labeled by fluorescence energy donor NBD and energy acceptor rhodamine. These two probes fulfill the spectroscopic criteria of fluorescence resonance energy transfer when they are in close proximity. Therefore, the assembly of a peptide can be determined with the help of energy transfer experiment between its NBD- and rhodamine-labeled analogues. Panel A of Fig. 6 shows the fluorescence spectra of energy transfer experiments between NBD-H-205 and Rho-labeled H-205 in PC/PG/Chol lipid vesicles. Fluorescence spectra of NBD-H-205 in PC/PG/Chol vesicles were recorded in the presence of increasing amounts of Rho-H-205. As shown in the panel A of Fig. 6, the addition of energy acceptor Rho-H-205 to the membrane-bound donor NBD-H-205 resulted in an appreciable decrease in NBD fluorescence concomitant with the increase in rhodamine fluorescence. This observation was consistent with the energy transfer from NBD-labeled H-205 to Rho-labeled H-205 and suggested that H-205 self-assembled in PC/PG/Chol lipid vesicles. Appreciable energy transfer was observed between NBD-H-205 and Rho-H-205 in PC/Chol vesicles (profiles not shown, data shown in panel C), which indicated that this synthetic leucine zipper-like motif also assembled in zwitterionic lipid vesicles. Panel B of Fig. 6 depicts the results of energy transfer experiments between NBD-Mu1-H-205 and Rho-Mu1-H-205 in PC/PG/Chol vesicles. In contrast to the wild-type peptide, fluorescence of the donor peptide decreased only a little as a result of the addition of the acceptor peptide Rho-Mu1-H-205. These results clearly suggested that the mutant peptide Mu1-H-205 did not self-assemble in PC/PG/Chol vesicles, and therefore, an appreciable amount of energy transfer was not observed between the NBD- and Rho-labeled Mu1-H-205. Also no significant energy transfer was observed between NBD-Mu2-H-205 and Rho-Mu2-H-205 in PC/PG/Chol lipid vesicles. Similar energy transfer results were obtained with the mutant peptides in PC/Chol vesicles indicating the inability of the mutant peptides to assemble in PC/Chol vesicles. In order to confirm that the observed energy transfer was due to the assembly of the peptides, the percentages of energy transfer with different pairs of energy donor and acceptor were compared with that of the randomly distributed energy donor and acceptor as described earlier (49, 50, 22). Panel C of Fig. 6 shows such plots, which clearly indicates that the energy transfer efficiencies of NBD-H-205 and Rho-H-205 either in PC/Chol or PC/PG/Chol vesicles are much above the random distribution level. However, energy transfer efficiencies of other pairs are close to the random distribution level.

Wild-type H-205 Co-assemblies with Another Leucine Zipper-like Motif H-130 in Aqueous Solution—In order to explore for further possible roles of the H-205 segment in the assembly of hemolysin E in aqueous environment, its self- and co-assembly with other similar motif were tested in aqueous buffer. For this purpose fluorescence and circular dichroism approaches were employed as reported earlier (45). In order to check the ability of H-205 to self-assemble, its Rho-labeled analogue was treated with proteinase-k. However, very little de-quenching of rhodamine fluorescence was observed in the concentration range of 0.075–0.60 μM, which indicated that Rho-H-205 was probably not aggregated in solution in this concentration range. This is in contrast to the Rho-labeled leucine zipper motif derived from viral fusion protein, which is aggregated and significantly de-quenches the fluorescence at this concentration range after treatment with proteinase-k (45). The possibility of co-assembly of H-205 with other leucine zipper-like motif in HlyE was also checked. Crystal structure of the water soluble form of hemolysin E (10) shows the coiling between helices. One such coiling is visualized between the C-terminal of αC and the N-terminal of αF (10). Therefore, it was of interest to check if H-205, which is derived from the N-terminal of αF, can interact with another peptide H-130 (amino acid 130–157), derived from the C-terminal of αC. In order to get evidence about this co-assembly, fluorescence spectra of their Rho-labeled analogues were recorded both individually and in mixture. It was
observed that the rhodamine fluorescence of the mixture of Rho-labeled H-205 and H-130 was ~30% less than the expected algebraic sum of their individual fluorescence levels (panel A, Fig. 7). Interestingly, the addition of proteinase-k to the mixture caused an increase in fluorescence, which further brought the fluorescence level close to their algebraic sum. It is important to mention that at this concentration (0.175 μM), addition of proteinase-k to Rho-H-205 resulted only in a 3–4% increase in rhodamine fluorescence while for Rho-130 the increase was ~5–8% (panel A). However, the rhodamine fluorescence of the mixture of Rho-Mu2-H-205 and Rho-H-130 was very close to the algebraic sum of their individual fluorescence levels and addition of proteinase-k to their mixture caused only a little increase in fluorescence. These results probably indicate that the decrease in fluorescence of Rho-H-205 after mixing with Rho-H-130 is due to the specific co-assembly between them. In order to get further evidence, similar experiments were carried out by circular dichroism technique. Interestingly, it was observed that circular dichroism of the mixture of H-205 and H-130 had significantly more negative ellipticity values than the algebraic sum of the two individual spectra (panel B). However, when mutant peptides (Mu1-H-205 or Mu2-H-205) were used instead of the wild-type H-205, the algebraic sum of two individual spectra was very close to that of their mixture (panel C). These CD results further support the sequence-specific co-assembly between H-205 and H-130 peptides.

**Wild-type H-205 Destabilize the Phospholipid Bilayer More Efficiently than the Mutant Peptides**—In order to determine whether this amphipathic leucine zipper-like motif has any role in destabilizing the target membrane or not, peptide-induced dissipation of diffusion potential experiments were performed as reported earlier (30–34,22) and described under “Experimental Procedures.” Peptides were added to the negatively charged PC/PG/Chol or zwitterionic PC/Chol lipid vesicles, pretreated with potential sensitive fluorescent dye, DiSC3(5) and K+-ionophore valinomycin. Panel A of Fig. 8 shows some experimental profiles of wild-type H-205 and mutant peptide Mu2-H-205. As shown in panel A, the addition of H-205 to the PC/PG/Chol lipid vesicles, resulted in the fluorescence recovery of the dye due to dissipation of diffusion potential across the membrane in a dose-dependent manner. This peptide-induced fluorescence recovery further indicated that H-205 could permeate PC/PG/Chol membrane. It is interesting to note that at a lower peptide concentration fluorescence recovery exhibited a slower kinetics (a, b), which became significantly faster at higher concentration (c–e). However, both the mutants were inactive in inducing any fluorescence recovery indicating their inactivity to permeate PC/PG/Chol lipid vesicles. Panel B of Fig. 8 shows the plot of peptide-induced fluorescence recovery with respect to peptide/lipid molar ratio. Peptide induced dissipation of diffusion potential was also checked in zwitterionic PC/Chol lipid vesicles. H-205 was less active in inducing membrane permeability in PC/Chol vesicles compared with that in PC/PG/Chol as shown in the panel C of Fig. 8. However, the mutant peptides were less active than the wild type in PC/Chol vesicles. These results indicate that the wild-type H-205 induced ion permeation in both PC/Chol and PC/PG/Chol lipid vesicles and was more active than the mutant peptides.

In order to further characterize the membrane permeability of H-205 and its analogues, release of calcein from calcein entrapped lipid vesicles in the presence of these peptides were monitored. As shown in panels A and B of Fig. 9 the synthetic leucine zipper-like motif (H-205) triggered the release of liposome-encapsulated calcein in a concentration-dependent manner (Fig. 9). Similar to the dissipation of diffusion potential experiment, H-205 was more active in negatively charged PC/PG/Chol than only zwitterionic PC/Chol lipid vesicles. However, the activity of H-205 was very much sequence-specific, as both the mutants were less efficient than the wild type in releasing calcein from either of the negatively charged or zwitterionic lipid vesicles. *Inset of panel A* of Fig. 9 gives a comparison of the activity of the wild type and the mutant peptides in releasing liposome-encapsulated calcein and their kinetics in negatively charged PC/PG/Chol lipid vesicles.

**DISCUSSION**

We have identified a conserved leucine zipper-like motif (amino acid 205–234) near the C-terminal of the protein-toxin hemolysin E. The results shown here indicate that the peptide
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charged lipid vesicles. Titration experiments (Fig. 3) with lipid vesicles indicated the higher partition co-efficient of NBD-labeled H-205 for PC/PG/Chol than PC/Chol lipid vesicles. This result reveals the role of electrostatic interaction between the positive charges of the amino acids and negative charges of the phosphatidyl glycerol (PG) head groups in determining the affinity of this peptide toward the phospholipid vesicles. It is noteworthy that this segment has five net positive charges. Also it was observed that both the mutant peptides with the same number of positive charges exhibited lower partition co-efficients than the wild-type peptide in PC/PG/Chol vesicles indicating that mutations did affect the affinity of the peptides for negatively charged lipid vesicles although didn’t inhibit their binding onto it. Furthermore, the results suggest the role of specific sequence in determining the affinity of this synthetic motif toward the negatively charged PC/PG/Chol lipid vesicles.

Proteolytic cleavage experiments (Fig. 4) revealed that the wild-type NBD-labeled peptide appreciably resisted the cleavage by proteinase-k when it was bound to negatively charged lipid vesicles indicating the possibility of insertion of the peptide in the lipid bilayer in contrast to the mutant peptides, which were located on the surface of the lipid vesicles and hence were cleaved promptly by the enzyme. These results indicate the role of specific sequence in localization of this synthetic motif in the negatively charged lipid vesicles. However, the NBD-labeled wild-type H-205 and the mutant peptides were cleaved promptly by proteinase-k when they were bound to zwitterionic lipid vesicles. The results of the proteolytic cleavage experiments were supported by the characteristic emission spectra of NBD-labeled wild type and mutant peptides in negatively charged and only zwitterionic lipid vesicles. The observed emission maximum of around 526–527 nm exhibited by NBD-H-205 in negatively charged lipid vesicles (Fig. 2) was much shorter than that observed for NBD-probe (533 nm) located on the surface of the membrane (47). Whereas, emission maxima exhibited by NBD-H-205 in zwitterionic (529–530 nm) and two NBD-labeled mutant peptides (531–532 nm) in either of the negatively charged or zwitterionic lipid vesicles were close to the characteristic emission maximum of the probe, located onto the membrane surface.

The shape of the binding isotherm of NBD-H-205 suggests that it binds cooperatively and forms large aggregates in negatively charged lipid vesicles (Fig. 3, panel B). The similar downward bending of binding isotherms was observed in case of membrane binding of channel-forming peptide toxin pardaxin (30) and alamethicin (48). In contrast to the wild-type peptide, the binding isotherms (panel C) of NBD-labeled mutants in negatively charged or zwitterionic lipid vesicles were linear in nature indicating their inability to form large aggregates in these kinds of vesicles. However, similar to the mutant peptides, H-205 did not form large aggregate in only zwitterionic lipid vesicles.

Leucine zipper motifs are known to adopt helical structure. Crystal structure of the water-soluble form of hemolysin E indicated helical structure for this part of the protein (10). CD experiments done in this study suggest that H-205 although did not exhibit appreciable helical content in aqueous environment, it adopted significant helical structure in membrane-mimetic environments like SDS micelle (1% v/v in water) and TFE/water (40% v/v). However, both the mutant peptides exhibited less helical structure than the wild-type H-205, indicating that the mutations affected the secondary structure of this synthetic leucine zipper-like motif. Although nothing is known about the secondary structure of the toxin in membrane, the results further indicate that probably this segment may contribute in maintaining the overall secondary structure of the protein in membrane.
Energy transfer experiments clearly demonstrated that only the wild-type H-205 but not its mutants could self-assemble in both PC/PG/Chol and PC/Chol lipid vesicles. These results do not contradict the binding experiment data, which suggest that the wild-type H-205 forms large aggregates only in negatively charged PC/PG/Chol vesicles. Combining the membrane binding and the energy transfer results, it can be stated that although H-205 self-assemble in both negatively charged and zwitterionic vesicles, it forms large aggregates only in negatively charged lipid vesicles. The mutant peptides distributed randomly in either of the lipid vesicles indicating their inability to self-recognize and assemble in these lipid vesicles. The results further suggest the importance of amphipathicity and heptadic amino acid in determining the assembly of this synthetic leucine zipper-like motif in membrane. Proper assembly of any pore-forming protein is an essential criterion for its functional activity. Leucine zipper motifs are known to play crucial role in the assembly of a protein/polypeptide in aqueous and membrane environments as well. The sequence specific assembly of wild-type H-205 in both negatively charged and only zwitterionic lipid vesicles further raises the possibility that this segment may assist in the oligomeric assembly of membrane...
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hemolysin E in the target cell membrane. Moreover, this finding is consistent with the observation that several hemolysin E monomers assemble in membrane to form pore (10). The observation that H-205 although assemble in both kinds of vesicles but it forms large aggregates only in negatively charged lipid vesicles, indicates the importance of both lipid composition and the amino acid sequence in determining the nature of assembly of a peptide in phospholipid vesicles. Interestingly, despite having the same number of positive charges the mutant peptides did not self-assemble in negatively charged lipid vesicles.

H-205 segment does not appreciably aggregate in solution as evidenced by the fluorescence study of Rho-labeled peptides in the presence of proteinase-k. However, both circular dichroism and fluorescence studies revealed that H-205 co-assembled with another leucine zipper-like motif (H-130), derived from the C-terminal of αC (Fig. 7). This result is consistent with the crystal structure of hemolysin E (10), which shows coiling of helical segments including these two leucine zipper-like motifs. This is an interesting observation that two synthesized segments can exhibit a segment-segment interaction that exists in the whole protein. Furthermore, the co-assembly between these two leucine zipper-like motifs is in agreement with the mostly monomeric nature of the molecule in aqueous environment (10). Probably, the results indicate that H-205 and H-130 motifs are involved in the intramolecular segment-segment interaction to assist in the monomeric assembly of HlyE in aqueous environment.

Since hemolysin E is a pore-forming toxin, it was of interests to check if these synthetic peptides derived from the leucine zipper motif could destabilize the lipid bilayers. Dissipation of diffusion potential experiments (Fig. 8) clearly indicated that H-205 peptide could permeate both negatively charged (PC/PG/Chol) and electrically neutral (PC/Chol) vesicles. However, the membrane permeation activity of H-205 was lower in PC/Chol than that of in PC/PG/Chol lipid vesicles. The mutant peptides were less active than the wild type in both negatively charged and zwitterionic lipid vesicles. Membrane destabilization by pore formation usually maintains certain criteria (51–54). First, binding of monomeric peptides to the membrane in α-helical structure, secondly, assembly of monomeric peptide on the surface of the membrane, thirdly, insertion of the peptide helices into the hydrophobic core of the membrane and lastly, progressive recruitment of more peptide monomers to increase the pore size. The results discussed above indicated that H-205 adopted helical structure in membrane mimetic environments. Energy transfer and membrane binding experiments suggested that H-205 self-assembled and formed large aggregates in negatively charged lipid vesicles. H-205-induced membrane permeation in negatively charged lipid vesicles exhibited a faster kinetics at higher peptide concentration. The result probably indicates that organization of H-205 in negatively charged lipid vesicles is concentration dependent and at higher concentration it facilitates the passage of more ions at a time. Thus all these data support in favor of pore formation of H-205 in negatively charged PC/PG/Chol lipid vesicles. Consistent with this possibility this synthetic leucine zipper-like motif efficiently induced the release of calcine from the calcine-entrapped negatively charged lipid vesicles (Fig. 9). Inactivity of the mutant peptides to induce appreciable membrane permeation, indicate the role of amphipathicity and heptad leucine in the functional activity of the peptide. However, another possibility (36) cannot be ruled out in which a helical amphipathic H-205 segment is tightly bound to the acidic phospholipid head groups and self-assemble with other peptide monomers. This aggregate is probably oriented in such a way that its hydrophobic face is inserted shallowly within the hydrophobic core of the membrane and hydrophilic face directed toward the charged lipid head groups. In such assembly, H-205 might be protected from proteolysis and could perturb the membrane integrity. Since H-205 cannot form large aggregates in PC/Chol vesicles and located onto its surface, probably pore-formation was not involved with the H-205-induced destabilization of zwitterionic phospholipid membrane.

Based on hydrophobicity analysis (2), site-directed mutagenesis (9) and model building studies (10), it has been proposed that the β tongue consisting of a β hairpin and two short helices oD and αE is the transmembrane pore-forming segment of hemolysin E. However, the precise membrane-interacting domains and the assembly of HlyE in membrane are not yet known. The amphipathic leucine zipper-like motif (amino acid 205–234) is located at the N-terminal of helix αF, which is just adjacent to the proposed β tongue region of hemolysin E. Amphipathic leucine zipper motifs have often been implicated to important structural and functional roles in membrane proteins for example in binding of viral fusion proteins to the target cell membrane and their assembly in aqueous and membrane environments (55–58, 22, 45). Our observations that the synthetic peptide (H-205) derived from the leucine zipper motif binds to both mixture of zwitterionic and negatively charged PC/PG/Chol and only zwitterionic PC/Chol lipid vesicles and assembles therein with sequence specificity suggest a probable role for this motif in binding and assembly of the toxin in the target cell membrane. It has been demonstrated that H-205 can permeabilize both zwitterionic and negatively charged lipid vesicles. The results further raised a possibility that this leucine zipper-like motif in hemolysin E may assist in the destabilization of the target cell membrane.

However, the observations that H-205 although binds to and assembles in both zwitterionic (PC/Chol) and negatively charged (PC/PG/Chol) lipid vesicles but destabilizes the negatively charged lipid vesicles more efficiently, forms large aggregates and inserts only in negatively charged but not in zwitterionic lipid vesicles are interesting. These results probably suggest that the amino acid sequence requirements for a peptide to effectively destabilize the negatively charged vesicles are different from that for only zwitterionic lipid vesicles. Furthermore, the results presented here are in support of increasing evidence that α-helical integral membrane proteins can serve as structural elements and assist in the organization and assembly of the protein in phospholipid membrane (59–63).

In conclusion, we have identified a conserved leucine zipper-like motif in hemolysin E, which has the potential to participate in the binding of the toxin to the target cell membrane. Moreover, this segment may be involved in the assembly and toxin induced destabilization of target cell membrane. We speculate that this conserved motif may have similar structural and functional roles in homologous proteins of this family.

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