CHAPTER—3

STRUCTURE-FUNCTION CORRELATION IN δ-TOXIN AND ITS ANALOGS CORRESPONDING TO CARBOXYL TERMINAL REGION
3.1. Introduction

Hemolytic and antimicrobial peptides such as melittin (DeGrado et al., 1982; Tosteson et al., 1985; Dempsey, 1990), alamethicin (Latorre and Alvarez, 1981; Hall et al., 1984), gramicidin A (Urry 1971; Anderson, 1984), pardaxin (Shai et al., 1988; Shai, 1994), magainins (Zasloff, 1987; Matsuzaki et al., 1989; 1991) and cecropins (Steiner et al., 1981; Christensen et al., 1988) exert their biological activities by permeabilizing membranes. However, the peptides show no similarity with respect to primary structure, amino acid composition and charge (Saberwal and Nagaraj, 1994; Maloy and Kari, 1995; White et al., 1995; Bechinger, 1997; Nagaraj, 1997; Matsuzaki, 1998). Alamethicin and gramicidin A have non-coded amino acids like α-aminoisobutyric acid and D-amino acids, respectively. Although, the above-mentioned peptides permeabilize membranes, the manner in which they permeabilize is different. Gramicidin A forms channels which allow the passage of monovalent cations (Killian, 1992; Anderson et al., 1999) whereas alamethicin forms channels composed of helical peptide monomers (Nagaraj and Balaram, 1981; Sansom, 1991; Cafiso, 1994). Although magainins and cecropins alter the conductance of planar bilayers (Ducloheir et al., 1989; Cruciani et al., 1991; Matsuzaki et al., 1997; Silvestro et al., 1997; Wang et al., 1998), the channels are not characterized at a molecular level.

The physico-chemical properties of peptides that determine the interaction with lipids have been the subject of extensive investigations. It appears that several factors such as hydrophobicity, amphiphilicity and net charge play an important role in determining membrane association and extent of partitioning (Zasloff, 1987; Saberwal and Nagaraj, 1994; Dathe and Wieprecht, 1999; Sitaram and Nagaraj, 1999). It also appears that reduction in length often does not result in loss of binding to membranes or biological activities and in some cases modulates biological activities (Merrifield et al., 1982; Boman et al., 1989; Sitaram and Nagaraj, 1990). For example, in melittin and pardaxin shorter peptides have been obtained with selective antibacterial activity unlike their parent peptides, which are non-specific lytic agents (Thennarasu and Nagaraj, 1995; 1996; Subbalakshmi et al., 1999). Also, these short peptides derived from the longer peptides do bind to membranes and cause permeabilization (Sitaram and Nagaraj, 1990; Sitaram et al., 1997).

In order to get an insight into the biophysical properties that are important for the hemolytic activity of δ-toxin, an approach involving synthesis of δ-toxin analogs, structural
investigation by CD spectroscopy and membrane interaction studies were undertaken. The results are described in this chapter.

3.2. Results

3.2.1. Choice of peptides

Nuclear magnetic studies by Lee et al., (1987) and Tappin et al., (1988) have indicated that the amino acids 2-20 of δ-toxin form a relatively stable helix whereas the C-terminal end is flexible. In an attempt to determine the relative contributions of the different segments of δ-toxin towards structure and activity, peptides corresponding to residues 5-20, 11-26 as well as systematic deletion from the N-terminus were generated by chemical synthesis. The primary structures of the peptides used for studies are summarized in Table 3.1 along with the charges they would possess at neutral pH. The secondary structures of the peptides were examined by circular dichroism spectroscopy.

The structures of peptides spanning the helical segment 5-20 (16D), the helical and flexible portion 11-26 (16P) and δ-toxin (26P) as determined by CD spectroscopy in different environments are shown in Fig.3.1. A-C. In aqueous medium, 16D and 16P show spectra characteristic of unordered conformations unlike 26P (Fig. 3.1.A) which has a spectrum characteristic of peptides in helical conformation, with a helical content of ~61% (estimated using the equation θ_{222} for 100% = [n - 4.6] X - 40,000 / n; Gans et al., 1991; Manning and Woody, 1991). In aqueous methanol (Fig. 3.1.B) and TFE (Fig. 3.1.C) the peptides, 16D and 16P show preference for helical conformations. The ellipticity value at θ_{222} indicates an α-helical content of 35% for 16D, 70% for 16P and 76% for 26P in TFE. Thus, 16D has a lower helical content than 16P or 26P. Interestingly, 16P has a helical content comparable to the entire toxin, although it is composed of helical and unordered segments. In the presence of DOPC vesicles, 16P has greater helical content as compared to 16D and the helical content is similar to 26P (Fig.3.2.A). In DOPE:DOPG (3:1) vesicles, 16P has helical content comparable to 26P (Fig.3.2.B). The nature of lipid does not appear to dictate the conformation of 16D, as the CD spectra in zwitterionic and anionic lipids are similar. The CD data indicates that in isolation, the segment DLVKWIIDTVNKFTKK that has 10 out of 16 residues in ordered helical conformation and rest in flexible conformation in δ-toxin (the underlined residues correspond to helical conformation in δ-toxin as determined by NMR) has helical propensity comparable to the entire toxin. However, the segment IISTIGDLVKWIIDTV which is
Table 3.1. Sequences of synthetic peptide analogs corresponding to C-terminal region of δ-toxin.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Charge at neutral pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>26P</td>
<td>for-MAQDIISTIGDLVKWIIDTVNKFTKK</td>
<td>0</td>
</tr>
<tr>
<td>-f26P</td>
<td>MAQDIISTIGDLVKWIIDTVNKFTKK</td>
<td>+1</td>
</tr>
<tr>
<td>25P</td>
<td>AQDIISTIGDLVKWIIDTVNKFTKK</td>
<td>+1</td>
</tr>
<tr>
<td>23P</td>
<td>DIISTIGDLVKWIIDTVNKFTKK</td>
<td>+1</td>
</tr>
<tr>
<td>23K</td>
<td>KIISTIGDLVKWIIDTVNKFTKK</td>
<td>+3</td>
</tr>
<tr>
<td>22P</td>
<td>IIISTIGDLVKWIIDTVNKFTKK</td>
<td>+2</td>
</tr>
<tr>
<td>20P</td>
<td>STIGDLVKWIIDTVNKFTKK</td>
<td>+2</td>
</tr>
<tr>
<td>18P</td>
<td>IGDLVKWIIDTVNKFTKK</td>
<td>+2</td>
</tr>
<tr>
<td>16P</td>
<td>DLVKWIIDTVNKFTKK</td>
<td>+2</td>
</tr>
<tr>
<td>16D</td>
<td>IISTIGDLVKWIIDTV</td>
<td>-1</td>
</tr>
</tbody>
</table>

<sup>a</sup> charged amino acids are in bold letters.
Fig. 3.1. CD spectra of peptides 16D, 16P and 26P in different media.  
A) Aqueous buffer pH 7.4,  B) Aqueous methanol (1:1) and C) TFE.  
The spectra were recorded at a peptide concentration of 10 μM.
Fig. 3.2. CD spectra of peptides 16D, 16P and 26P in liposomes. 
A) DOPC and B) DOPE:DOPG (3:1) at a peptide lipid molar ratio of 1:100. Peptide concentration was 8 μM.
entirely helical in δ-toxin has lower propensity for helical conformation in isolation. The tendency to form helical conformation for 16P and 16D is apparent only in media of low dielectric constant whereas in aqueous medium both the peptides are in unordered conformation. However, δ-toxin shows high propensity for helical conformation even in aqueous medium. Since, 16P has greater propensity for helical conformation in organic solvents and lipids as compared to 16D, the peptides of increasing lengths from the C-terminus have been synthesized (sequences are summarized in Table 3.1) and analyzed for conformational propensities and activity.

3.2.2. Secondary conformations of peptides

The CD spectra of various peptides in aqueous medium are shown in Fig.3.3. A – F. No concentration dependent changes in CD spectra are observed for 16P, 18P and 20P in the range 2-100 μM. The spectra indicate predominately unordered conformation. In peptide 22P, there is a perceptible increase in helical content as judged by an increase in -θ222 as a function of peptide concentration with a maximum -θ222 value observed at 32 μM and no increase in helical content was observed beyond this concentration. 25P is helical even at low concentrations and there is no concentration dependent increase in helical content above 4 μM while, the spectra observed at 6 μM is virtually same. However, in -f26P (where the N-terminus is not blocked by formyl group) an increase in helical content observed from 0.5 to 4 μM. Also, the helical contents of 25P and -f26P are comparable. It thus appears that at least 22 (deletion of four N-terminal amino acids in the toxin) of the 26 residues of δ-toxin are needed for helix formation in aqueous environment. Infact, no concentration dependent increase in -θ222 was observed in 23P in the concentration range beyond 5 μM and the helical contents are similar as observed for 25 and 26 residue peptides. The concentration dependent CD spectra for 26P observed in this study and as reported by Thiaudiere et al., (1991) are identical in aqueous medium. The spectrum of 23P as a function of concentration is shown in Fig.3.4.A. Thus, introduction of D at the N-terminus of 22P appears to favor formation of stable helical structure in aqueous medium. The spectra of the peptides in TFE are shown in Fig.3.5. Interestingly, all the peptides show similar spectra. Also, in DOPC (Fig.3.6) and DOPE:DOPG [3:1] (Fig.3.7) lipid vesicles, all the peptides adopt α-helical conformation. Estimation of helical contents from -θ222 values indicates that all the peptides have ~70 – 75% helical content. This could be interpreted as either 70% population in all the peptide
Fig. 3.3. Concentration dependent CD spectra of peptides in aqueous media.
A) 16P, B) 18P, C) 20P, D) 22P, E) 25P and F) f26P.

The peptide concentrations are mentioned against each spectra.
Fig. 3.4. Concentration dependence CD spectra of peptides.
A) 23P in aqueous medium  B) 23K in aqueous medium
The peptide concentrations are mentioned against each spectra.
Fig.3.5. CD spectra of peptides in TFE.
The peptide concentration was 10 μM. Peptides are labeled against each CD spectra.
Fig. 3.6. CD spectra of peptides in DOPC liposomes.
The spectra were recorded at a peptide lipid molar ratio of 1:100 and the peptide concentration was 8 μM. Peptides are labelled against each spectra.
Fig. 3.7. CD spectra of peptides in DOPE:DOPG (3:1) liposomes. Spectra were recorded at a peptide lipid molar ratio of 1:100 and the peptide concentration was 8 μM. Peptides are labeled against each spectra.
molecules adopt helical conformation or 70% of the residues in a peptide molecule fold in to helical conformation. At the concentration of 8 µM, 16P, 18P and 20P are largely unordered in aqueous medium but fold into helical conformations in presence of DOPC and DOPE:DOPG (3:1) vesicles whereas no increase in helical content were observed for 25 and 26 residue peptides. The ellipticity value for the peptides in DOPC vesicles at 200 nm is relatively less as compared to aqueous medium, TFE and DOPE:DOPG (3:1) vesicles. It is possible that there is a conformational heterogeneity in DOPC vesicles with a fraction of molecules populating β-conformation whereas in DOPE:DOPG (3:1) vesicles the spectra are characteristic of helical conformation (Fig.3.7).

In order to examine the effect of pH on structure of f26P, spectra were recorded at pH 4.0, pH 10.2 and the data is shown in Fig.3.8. The spectra at low (Fig. 3.8.A), neutral (Fig.3.3.F) and high pH (Fig.3.8.B) are virtually identical in the concentration range 5 – 6 µM and the helical contents are same indicating that there is no effect of pH on the formation of helical structures.

3.2.3. Hemolytic activity of δ-toxin and its synthetic peptides

The concentration dependent hemolytic activity of peptides (listed in Table 3.1) on erythrocytes of guinea pig is shown in Fig. 3.9. Peptide 22P is most active peptide followed by the peptides 20P, f26P, 26P (native δ-toxin), 25P, 18P and 16P. Peptide 16D exhibits <10% hemolysis at 150 µM. The results shown in Fig. 3.9. indicate that the lytic potency varied significantly among the different peptides. 16D comprising of residues 5-20 corresponding to the α-helical region of δ-toxin is inactive and 16P corresponding to residues 11-26 of δ-toxin is considerably less hemolytic than the longer peptides. 18P, which is an extension of 16P by two amino acids at the N-terminus, is more active than 16P. Introduction of residues, S and T to 18P results in considerable increase in hemolytic activity. Like wise 22P, which is 20P plus two I residues is the most active peptide. Interestingly, δ-toxin (26P) is less active as compared to 20P and 22P. Formylation at the N-terminus does not appear to be an important requirement for activity as the hemolytic activity shown by f26P and 26P are same. The N-terminal M of δ-toxin appears to modulate activity, as 25P is less active than f26P. Though net cationicity of shorter peptides is an important determinant for hemolytic activity, it is unlikely that decrease in hemolytic activity observed for 25P, f26P and 26P could be due to decreased cationicity as compared to 22P. The data indicates that maximum
Fig. 3.8. Concentration dependence CD spectra of $\alpha$26P. 
A) in Sodium citrate buffer pH 3.95 B) in Sodium borate buffer pH 10.20. Peptide concentrations were 2.8, 4.2, 5.5 and 7.6 μM.
Fig. 3.9. Hemolytic activity of peptides.

The hemolytic activity of δ-toxin and its generated peptide analogs monitored against guinea pig erythrocytes in an isotonic buffer. The hemolysis was measured after incubating erythrocytes with peptides for half an hour at 37°C and the measured the OD at 540 nm.
activity is obtained in the peptide corresponding to residues 5-26, which is composed of helical and flexible region of δ-toxin. Interestingly, at the concentration at which maximal lysis is observed, 22P is only partially helical with a large fraction of molecules populating unordered conformations and the similar pattern is observed for 20P.

The average hydrophobicity and GRAVY hydrophobic index calculated for the peptides using the PCGENE program are summarized in Table 3.2. This analysis indicates that 22P is the most hydrophobic peptide followed by peptides 26P and 25P correlating with the observation that 22P has the greatest hemolytic activity. However, 20P is more active than 25P and 26P which would not be expected on the basis of hydrophobic index. The FPLC elution pattern on reverse phase column does not correlate with the hydrophobic indices shown in Table 3.2. However, the retention times do correlate with the propensity to adopt helical conformations in aqueous media, they do not directly correlate with hemolytic activity. The peptides and proteins that interact with the surfaces in an orientation specific manner via a specific hydrophobic contact area, change the hydrophobic contact area which in turn alter the binding properties of peptides (Blondelle et al., 1992; 1995; Hodges et al., 1994; Lazoura et al., 1997).

3.2.4. Fluorescence studies and their aggregation behavior

Intrinsic fluorescence emission of the tryptophan residue is a simple and sensitive method to detect the local environmental changes in peptides and proteins (Lakowicz, 1983; Eftinik, 1991). The concentration dependent blue shift in tryptophan $\lambda_{\text{max}}$ of emission of the various δ-toxin fragments are shown in Fig. 3.10. All the peptides at lower concentrations showed an emission maximum of 351 ± 1 nm indicating that the tryptophan residue in these peptides is totally exposed to the aqueous environment. Peptides 16P, 18P did not show any shift in the emission maximum up to a concentration of 30 µM. There is a considerable blue shift of the emission maxima for the peptides 22P, 25P, -f26P and 26P. The maximal changes are observed for 25P. Interestingly, in δ-toxin the changes are less as compared to 25P and the toxin with free N-terminus (-f26P). The blue shift in the emission $\lambda_{\text{max}}$ suggests that that the tryptophan residues are shielded from aqueous environment probably as a consequence of peptide aggregation. Shift to the blue wavelength is observed even beyond the concentration where no further change in $\theta_{222}$ is observed in the CD spectra.
Table 3.2. Properties of peptides generated from C-terminal part of δ-toxin.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Average Hydrophobicity</th>
<th>Hydrophobic Moment</th>
<th>Hydrophobic Index GRAVY</th>
<th>% B required to elute</th>
</tr>
</thead>
<tbody>
<tr>
<td>16P</td>
<td>-0.05</td>
<td>0.65</td>
<td></td>
<td>52</td>
</tr>
<tr>
<td>18P</td>
<td>0.06</td>
<td>0.65</td>
<td>-0.17</td>
<td>55</td>
</tr>
<tr>
<td>20P</td>
<td>0.04</td>
<td>0.59</td>
<td>-0.90</td>
<td>61</td>
</tr>
<tr>
<td>22P</td>
<td>0.16</td>
<td>0.62</td>
<td>3.27</td>
<td>63</td>
</tr>
<tr>
<td>25P</td>
<td>0.10</td>
<td>0.61</td>
<td>0.80</td>
<td>72</td>
</tr>
<tr>
<td>-f26P</td>
<td>0.12</td>
<td>0.59</td>
<td>1.50</td>
<td>73</td>
</tr>
</tbody>
</table>

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$-----Retention times for elution of respective peptides from RP C-18 HR 5/5 column by FPLC using solvent mixture of A (0.1% TFA\H₂O) and B (0.1% TFA\CH₃CN) at linear gradient 0-100% of solvent B in 30 min as shown in Fig. 2.1. A.
Fig. 3.10. Concentration dependent tryptophan fluorescence of δ-toxin and its peptide analogs in aqueous media. The fluorescence emission maximum spectra were recorded by exciting tryptophan residues at 280 nm and the emission spectra monitored continuously from 300 - 400 nm.
3.2.5. **Membrane permeabilizing properties**

Membrane active peptides have the ability to form channels in the lipid membranes under the influence of transmembrane electrical potential (Sansom, 1991). This would lead to permeabilization of the lipid vesicles which results in the efflux of trapped solutes like CF and calcein (Kayalar and Duzgunes, 1986; Menestrina, 1988; Schwarz and Robert, 1990; Shai *et al.*, 1990; VazGomes *et al.*, 1993; Saberwal and Nagaraj, 1994). Extent of permeabilization has been correlated with the hemolytic activities. Release of entrapped CF from lipid vesicles composed of DOPC in the presence of δ-toxin and its 22, 25 residues peptide analogs is shown in Fig.3.11. The percentage release at different lipid-peptide ratios indicates that 22P is most effective in permeabilizing lipid vesicles followed by 26P and 25P. Similar results were observed for PC:Chl (7:3) liposomes. At a peptide lipid ratio of 1:100, CF release of 5%, 22%, 35%, 100%, 25% and 30% were observed for 16P, 18P, 20P, 22P, 25P and 26P peptides respectively after 50 seconds towards PC:Chl (7:3) liposomes. A double logarithmic plots of initial rate of CF release v/s concentration which yielded different slopes towards PC:Chl liposomes are shown in Fig. 3.12. Usually, efflux rates have been used to characterize the formation of pores in model membranes. Pore forming peptides initially bind, then insert in a trans-membrane orientation and finally aggregate in membranes to form a functional pore. The nature of the pore depends upon number of monomers in the pore complex. Pore forming proteins like, tetanus toxin (Menestrina *et al.*, 1989), *E.coli* hemolysin (Menestrina, 1988) and colicin E1 (Kayalar and Duzgunes, 1986) form the pore in monomeric form towards lipid vesicles as measured by CF efflux rates while, electric conductance measurement (Nagaraj *et al.*, 1980) and CF efflux experiments (Schwarz and Robert, 1990; 1992) for alamethicin have suggested a pore complex containing 6 monomers.

The smaller peptides like 16P, 18P which exhibit low hemolytic activity show a slope less than 1 whereas the most active peptide, 22P has a slope of ~ 6. Peptides 20P, 25P that exhibits lower hemolytic activity as compared to 22P have a slope in the range 1-2. The CF efflux studies indicate that 22P which exhibits greatest hemolytic activity is very efficient in permeabilizing the model membranes. It also suggests that a higher slope of 6 could be due to better ability of 22P to aggregate in model membranes involving 6 monomers to form a functionally active pore through which the inner cell contents leak out. It is likely that 22P has optimum structural requirements to aggregate in membranes but not in aqueous medium. Much of the structural transition, membrane permeabilization and hemolytic activity were
Fig. 3.11. Carboxyfluorescein release of peptides of 22P, 25P and 26P from DOPC liposomes.
The peptide lipid molar ratio are mentioned against each curve. The lipid concentration used was 100 μM.
Fig. 3.12. Double logarithmic plots of peptides.
Double logarithmic plots of initial rates v/s concentration of peptides A) 16P, B) 18P, C) 20P, D) 22P, E) 25P measured using 100 μM DOPC:Chl (7:3) liposomes. Initial rates were calculated by monitoring CF release for the first 20 seconds. The equation for the line and the regression values are mentioned in the box.
observed for 22P at 2 μM where it has very little secondary structure and do not has the ability to aggregate in aqueous medium. However, the CF efflux rates are dependent on nature of peptide and its concentration. The model membrane permeabilizing ability of the peptides parallels their hemolytic activity indicating membrane permeabilization by pore formation is the cause for hemolytic activity.

3.3. Discussion

The mechanisms that are involved in folding of proteins into their three dimensional structures are a subject that is being investigated extensively (Wiessman, 1995; Fersht, 1997; White and Wimley, 1999; Zamocky and Koller, 1999; Koide et al., 2000; Nolting and Andert, 2000). One approach that is becoming increasingly popular is the analysis of the solution conformations of peptide segments of protein that adopt either helical, β-sheet or γ-turn conformation in isolation (Fersht, 1997; Dobson and Karplus, 1999; Ohnishi et al., 2000). The general observation has been that such peptide segments are unordered in aqueous medium and tend to adopt secondary structures in structure-promoting solvents like TFE (Dyson et al., 1992; Kippan and Fersht, 1995; Reymond et al., 1997) and membrane media (Choung et al., 1988; Matsuyama and Natori, 1990; Sitaram and Nagaraj, 1993; Matsuzaki et al., 1997). NMR data in aqueous medium have been interpreted in terms of 'nascent' structures which could initiate folding of peptides (Dyson et al., 1988; Dyson et al., 1992; Bolin et al., 1996). These observations conform to the general observation that in aqueous medium, short peptides do not show preference for ordered conformations (Sunderlingam and Sekharudu, 1989; Dicapua et al., 1991).

A large number of peptides composed of 15-35 amino acids show a wide spectrum of biological activities (Boman, 1995; White et al., 1995; Nagaraj, 1997; Matsuzaki, 1999; Sitaram and Nagaraj, 1999). Structure determination of these bioactive peptides in order to correlate structure with activity and engineer them to get molecules with improved activity is a continuous challenge. Host-defense peptides from species across the evolutionary scale that have antimicrobial as well as cytolytic activities have been the subject of extensive structural investigations. The studies on these peptides are useful not only to rationalize the physico-chemical basis for their activity but also to design molecules that could have therapeutic potential (Andreu et al., 1992; Neu, 1992; Silver and Bostian, 1993). Bee venom hemolytic
peptide melittin has been and still is the subject of structure-function studies (Degrado et al., 1982; Benachir and Lafleur, 1995).

δ-hemolysin or δ-toxin from *S. aureus* is a 26-residue peptide with specific hemolytic activity (Fittin et al., 1980; Yianni et al., 1986) without antibacterial activity unlike melittin (Dhople and Nagaraj, 1993). While the presence of phospholipases could modulate the hemolytic activity of melittin segment (Dasseux et al., 1984), melittin devoid of phospholipase is important for proper evaluation of its biological activity. Similarly, the presence of α-hemolysin and β- hemolysin which are very potent lytic agents, (Alouf et al., 1989) could conceivably modulate the hemolytic activity of δ-toxin. Studies by Alouf et al., (1989) have shown difference in hemolysis when erythrocytes of different mammalian species were used. When synthetic δ-toxin was used, almost no lysis was observed with sheep erythrocytes whereas these cells were lysed to a significant extent by natural δ-toxin indicating that natural δ-toxin is more active than the synthetic one. As the sequence and conformation of both natural and synthetic δ-toxin are same, the discrepancy in their biological activity could be due to the contamination of natural δ-toxin with trace amounts of α- and β-toxins during purification process. As little as < 1% contamination of natural α- and β-toxins may account for discrepancy towards different species of mammalian erythrocyte lysis by natural δ-toxins. Hence, a synthetic approach was adopted to generate δ-toxin for proper evaluation of its hemolytic activity, determine structural requirements for activity and to examine the physico-chemical basis for its selective hemolytic activity. This approach would also help in identifying peptides composed of fewer amino acids as compared to the parent peptide that might have biological activity. Such short peptides could then be suitably engineered to enhance biological activity. Also, minimal structural requirements for membrane binding and biological activity can be delineated.

NMR studies have indicated that δ-toxin adopts α-helical conformation with residues 2-20 encompassing the helical region and the C-terminal 6 residue segment being relatively flexible (Tappin et al., 1988). The synthetic peptide corresponding to 5-20 segment composed of 16 residues is unordered in aqueous medium and tends to fold only in media of lower dielectric constant. The peptide corresponding to the segment 11-26 of δ-toxin, comprising both the helical and unordered regions is also unordered in aqueous medium. Interestingly, in media of low dielectric constant and lipid vesicles, this segment shows CD spectra which
indicates a helical content comparable to the entire toxin. Trifluoroethanol is known to induce helicity in peptides that are unstructured in aqueous medium (Zhong and Johnson, 1992; Kemmink and Creighton, 1995; Kippan and Fresht, 1995; Reymond et al., 1997). Therefore, detection of α-helices has been aided by use of TFE which induces the formation of helical structures in peptides and protein fragments that are mainly or partially unordered in aqueous solutions but have an intrinsic propensity to occur in helical conformations (Goodman et al., 1971; Kim et al., 1982; Brems et al., 1987; Dyson et al., 1988; Lehrman et al., 1990; Sonnichsen et al., 1992). However, the mechanism by which TFE stabilizes the structure is still debated upon, the studies suggest that TFE interacts preferentially with the peptide backbone to shift the structural equilibrium towards more ordered structure (Nelson and Kallbach, 1986; Lehrman et al., 1990; Shin et al., 1993; Jasanoff and Fresht, 1994; Shiraki et al., 1995). It has been proposed that hydrophobicity of the trifluoromethyl group and the ability to act as a strong hydrogen donor while being a weak acceptor are factors that help in stabilizing secondary structures of peptides (Goodwin et al., 1996; Rajan and Balaram, 1996). This solvent can also stabilize helices containing potential i, i+3 and i, i+4 salt bridges (Rajan and Balaram, 1996). Although 16P, 18P and 20P are unordered in aqueous medium, they adopt helical conformation in TFE with helical content comparable to the larger peptides 22P, 23P, 25P and 26P. The peptides composed of 22 amino acids and more show concentration dependent increase in helicity presumably as a result of aggregation driven by favorable hydrophobic interactions. In 16P and larger peptides ion-pair interactions between D, K residues at i, i+3 and i,i+4 positions is possible while these interactions appear to be stabilized in TFE but not in aqueous medium in 16P, 18P and 20P. Hence, in TFE, the peptides have high helical contents. In 16D, only one i, i+3 interaction is possible between D and K resulting in lower helical propensity in TFE compared to 16P.

It thus appears that in 26P, stabilization of helical structures in aqueous medium does not arise as a result of i, i+3 and i, i+4 interactions, otherwise helical structures would have also been observed in 16P, 18P and 20P. Since, there is an increase in hydrophobicity on going from 16P to 22P, association is driven by hydrophobic interaction which favors helical conformation. The propensity for helical conformation in 16P indicates that this segment contributes significantly to the helical structure of 26P. The helical structure in 26 residues peptide (-f26P) is stable as no changes in helical contents are observed at pH 3.95 and at pH 10.2. However, D at position 4 appears to be necessary for helical stability as replacement of
D by K results in considerable decrease in helical content (Fig. 3.4. B). It is possible that D at position 4 could be involved in intermolecular ion-pair interactions which are disrupted when D is replaced by K. Based on theoretical analysis, the model proposed by Raghunathan et al., (1990), suggests that six monomers are packed in an antiparallel manner involving D at position 4 in intermolecular charge interaction.

The amino acid requirements for a short peptide to adopt helical conformation in aqueous medium in the absence of aggregation have been extensively investigated (Marqusee and Baldwin, 1987; Marqusee et al., 1989; Padmanabhan et al., 1990; Armstrong et al., 1993; Padmanabhan and Baldwin, 1994; Scholtz et al., 1993). A hierarchy of amino acids, which favor helical conformation, has been observed (Chakrabarty et al., 1994; Zohu et al., 1994; Chakrabarty and Baldwin, 1995). However, in proteins and naturally occurring peptides like δ-toxin and melittin, the prediction of conformation by such rules may not be possible. Unlike in melittin, the C-terminal 16 residue segment has comparable propensity for helical conformation as the native peptide, especially in media of low dielectric constant.

The helical wheel representation of peptides 16P, 18P, 20P, 22P, 25P and 26 residue peptides are shown in Fig. 3.13. The hydrophobicity and hydrophobic moment for these peptides (Table 3.2) indicate that these peptides have the ability to exist as amphipathic helices (Eisenberg et al., 1982; Eisenberg, 1984) and thereby having strong tendency to aggregate due to tertiary interactions in order to adopt stabilized structures in aqueous media. Though very little is known about the stability of amphipathic helical peptides to exist as monomers in aqueous media, it has been suggested that hydrophobic interactions among the non-polar faces of the peptide helices can result in mutual stabilization (Padmanabhan and Baldwin, 1994) to form stable aggregates. Peptides that vary in length, sequence, amphipathicity, hydrophobicity can form aggregates of different states that have different pore or channel sizes which are responsible for hemolytic activity. Peptides generated on similar concepts have shown enhanced hemolytic activities than melittin (Blondelle et al., 1993; Comut et al., 1994).

A blue shift is observed in the emission $\lambda_{\text{max}}$ of tryptophan in peptides 22P, 26P, -f26P and 25P. Maximum blue shift is observed for 25P indicating that the tryptophan is in a more hydrophobic environment as compared to the other peptides. There is considerable difference in the environment of tryptophan in 26P and -f26P. Despite having similar helical content, the environments in tryptophan in 22P, 26P, -f26P and 25P in the aggregated forms
Fig. 3.13. Edmundson helical wheel representation of δ-toxin and its analogs corresponding to C-terminal region. Polar amino acids are underlined. Peptide sequences are mentioned in Table 3.1.
appear to be different with maximum hydrophobic environment observed in 25P and -f26P. Thus, the micro-environment of tryptophan can vary in different aggregated states. This is also reflected by the relative changes with an increase in fluorescence intensity as a function of peptide concentration.

Peptides 16P, 18P, 20P, 2P, 25P and the derivatives of 26P did not exhibit antibacterial activity against *E.coli* and *S.aureus*. When peptide 25P was used in model membrane permeabilization studies, it weakly permeabilized liposomes containing anionic lipids whereas the zwitterionic liposomes were permeabilized significantly at similar concentration. This peptide did not exhibit lysis of *E.coli* spheroplasts (Dhople and Nagaraj, 1993). However, the peptides have the ability to associate and adopt α-helical structures in presence of DOPE:DOPG lipid vesicles. Though all the peptides are amphipathic, they appear to be able to lyse only erythrocytes and not bacteria. Melittin and Paradaxin, which are hemolytic also, exhibit antibacterial activities unlike δ-toxin (Dempsey, 1990; Shai, 1994). This property of δ-toxin and its variants could arise as a result of the cationic residues 'not available' for interaction with the bacterial cell surface as a result of salt bridge formation with D residues. The only K residues that are available are the ones at positions 25, 26 in the C-terminal end. Since, they would not be a part of the amphilphic helix (as observed by NMR structure), such an arrangement does not favor, interaction with bacterial cell surface. Melittin also has a cluster of positive charges at the C-terminus. However, there are two R residues and two K residues, which favor effective interaction with bacterial membranes. The 13-residue peptide indolicidin, which has antibacterial and hemolytic activity, also has two R residues at the C-terminus (Ahmed *et al.*, 1995; Subbalakhsmi *et al.*, 1996).

Hemolytic activity arises as a result of the insertion of peptide into the lipid bilayer and perturbation of the lipid structure resulting in the formation of defects through which there can be flux of molecules resulting in colloid osmotic lysis. Alternately, peptides could aggregate and form transmembrane channels, through which solutes can move across. This process also results in colloid osmotic lysis. δ-toxin is presumed to cause lysis by formation of transmembrane channels. Now, for channel formation, aggregation as well as ability to span the lipid bilayer (~40 Å thickness) would be necessary. δ-toxin aggregates and the length of a helix composed of ~ 20 residues would be (~ 30 Å) sufficient to span the lipid bilayer. The structure of 16, 18, 20 residue peptides do not appear to aggregate. Also, the lengths of 16 and 18 residues would not be sufficient to span the lipid bilayer. Hence, they do
not exhibit lytic activity. However, 22P and 20P shows considerably greater activity than the parent δ-toxin. Now, the peptides appears to aggregate less effectively as compared to δ-toxin, hemolysis is observed at concentration at which the peptide is not aggregated. Peptide 23P, which has the ability to adopt α-helical conformations in aqueous medium similar to 25P would be expected to show lower hemolytic activity as compared to 22P. The results obtained by Kerr and co-workers showed that a peptide synthesized with a deletion of 3 amino acids from the N-terminus of δ-toxin (similar to 23P) exhibits a decrease in hemolytic activity and complete loss of channel forming ability as compared to δ-toxin. Therefore, it appears that the presence of peptides in a monomeric form in aqueous medium is more favorable for association with membranes than in the form of aggregates. Since, 22P and 20P do form aggregates in aqueous medium, membrane association can result in the formation of aggregates which can form transmembrane channels/pores. Although, non-hemolytic peptide 16P can adopt helical structures in lipid vesicles, the length would not be sufficient to span the lipid bilayer. Infact, these peptides exhibit weaker model membrane permeabilization ability as compared to active peptides.

In summary, the studies described in this chapter indicate that while even shorter segments of δ-toxin have the propensity to form helical structures in TFE and lipid vesicles, only the 25 and 26-residue peptides form helical structures in aqueous medium. The ability of these peptides to form helical structures appears to stem from their tendency to aggregate in aqueous medium which the shorter peptides do not show. However, the tendency to aggregate in aqueous medium is not essential for manifestation of hemolytic activity as the 20 and 22 residue peptides cause lysis more effectively than the parent peptides. Thus, the segment IISTIGDLVKWIIDTVNKFTKK appears to have the optimal hydrophobicity for exhibiting hemolytic activity. The lack of antibacterial activity appears to arise as a result of 'non availability' of positive charges for perturbing the bacterial membrane surface due to salt bridge formation when the peptides are in helical conformation.

The subsequent chapters of the thesis are devoted to engineer the peptides described in table 3.1 with specific emphasis on modulation of charges by selective replacement of D by K with a view to examine their effects on structure and biological activities. Peptides have also been synthesized with P in an effort to examine whether the helical structure is disrupted and its effect on biological activities.