Interaction of the 47-Residue Antibacterial Peptide Seminalplasmin and Its 13-Residue Fragment Which Has Antibacterial and Hemolytic Activities with Model Membranes

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ABSTRACT: The interaction of seminalplasmin (SPLN), a 47-residue antibacterial peptide, and its 13-residue fragment (SPF), which has antibacterial and hemolytic activities, with model membranes has been investigated. The fluorescence characteristics of the single Trp residue in these peptides indicate strong binding to lipid vesicles. SPLN binds more strongly to dioleoylphosphatidylglycerol vesicles compared to dioleoylphosphatidylcholine and phosphatidylserine vesicles. Localization studies using fluorescence quenchers like NO_3^-, I^-, and acrylamide indicate that the Trp residues in both of the peptides are located away from the head group region and are associated with the hydrophobic core. Both peptides cause release of carboxyfluorescein from zwitterionic as well as anionic vesicles. The biological activities of SPLN and SPF have been rationalized in terms of lipid–peptide interactions. It is proposed that the specificity in biological activity arises due to differences in the manner in which the peptides associate with the bacterial and red blood cell surfaces.

The biological activities of a large number of peptide toxins (around 20–35 residues long) have been rationalized in terms of the peptides having the ability to adopt amphiphilic α-helical structures and associate with lipid components of membranes (Bernheimer & Rudy, 1986; DeGrado et al., 1981; DeGrado, 1988; Kaiser & Kezdy, 1987). Most of these peptides that have antibacterial activity and hemolytic activity also strongly perturb model membranes whose lipid composition is similar to that of natural membranes. On association with membranes, many of these peptides are presumed to form ion channels resulting in the alteration of permeability and consequently cell lysis (Nagaraj & Balaram, 1981; DeGrado et al., 1981; Bernheimer & Rudy, 1986; Kaiser & Kezdy, 1987; Dempsey, 1990). Hence it is likely that the biological activity of these peptides stems from their ability to perturb the lipid bilayer structure of membranes and not by direct interaction with membrane proteins. Support for this argument comes from recent studies on synthetic melittin, magainin, and cecropins with all the amino acids in the D configuration. It has been shown that the all D peptides exhibit antibacterial and red blood cell (RBC)[1] lytic activity identical to that of the naturally occurring L forms (Wade et al., 1990; Bessale et al., 1990). Thus, the involvement of any chiral recognition in the biological activities of these peptides is ruled out. Seminalplasmin (SPLN) is a 47-residue peptide (Figure 1) (Sitaram et al., 1986) isolated from bovine seminalplasma, possessing antibacterial activity (Reddy & Bhargava, 1979). SPLN exerts its antibacterial activity by permeabilizing the bacterial inner membrane (Sitaram et al., 1992). A 13-residue peptide, SPF, corresponding to the most hydrophobic region of SPLN (Figure 1) has also been shown to have antibacterial activity (Sitaram & Nagaraj, 1990). However, this peptide, unlike SPLN, also has hemolytic activity. The antibacterial and hemolytic activities of SPF have been shown to arise from its ability to permeabilize the bacterial inner membrane and red blood cell membrane (Sitaram & Nagaraj, 1990). In order to find out whether the biological activities of SPF and SPLN arise due to their ability to perturb the lipid bilayer structure of membranes, we have investigated the interaction of SPF and SPLN with phosphatidylcholine lipid vesicles, phosphatidylserine lipid vesicles, phosphatidylglycerol and cardiolipin vesicles, and model membranes from lipids of *Escherichia coli* membranes. Our results indicate that the biological activities of SPLN and SPF can be rationalized in terms of lipid–peptide interactions.

Seminalplasmin

S D E K A S P D K H H R F S L S R Y A K L A N R L A N P K L L E T
1 10 20 30

F L S K W I G D R G N R S V
40

SPF

P K L L E T F L S K W I G
28 40

FIGURE 1: Primary structures of seminalplasmin (SPLN) and the 13-residue peptide SPF corresponding to residues 28–40.

MATERIALS AND METHODS

The synthesis and characterization of SPF has been reported earlier (Sitaram & Nagaraj, 1990). Synthetic SPLN (Sitaram et al., 1992) was used for the various investigations.

PS from bovine brain was purified by column chromatography on silica gel. DOPG, CL (bovine heart), and DOPC were from Avanti Polar Lipids (Birmingham, AL). The lipids were checked for purity by thin layer chromatography on silica gel. Small unilamellar vesicles (SUV) were prepared by sonication of a dispersion of lipids in 5 mM Hepes (pH 7.4) to clarity, in a Branson sonifier. Total lipids from *E. coli* were extracted by a modified method of Bligh and Dyer (Kates, 1972).

Peptide–lipid association was studied by monitoring the changes in the tryptophan fluorescence spectra of the peptides

[1] Abbreviations: CD, circular dichroism; CF, carboxyfluorescein; CL, cardiolipin; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PS, phosphatidylserine; RBC, red blood cell; SPF, peptide PKLLETFLSKWIG; SPLN, seminalplasmin.

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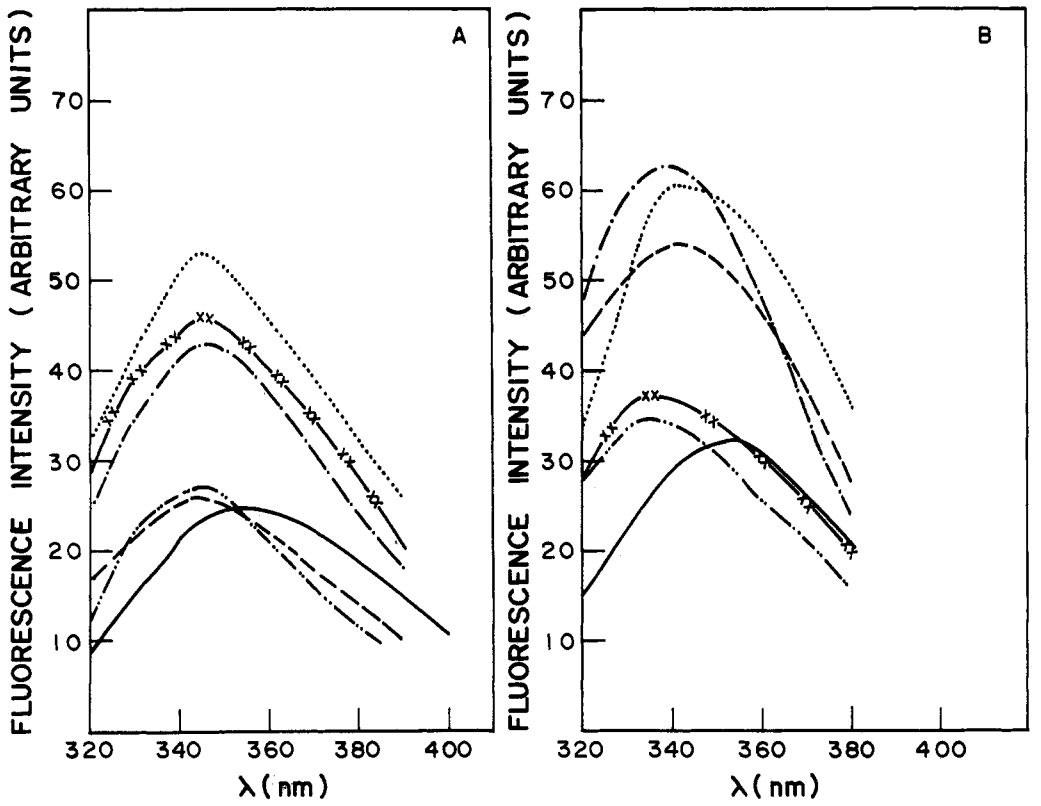

FIGURE 2: Fluorescence spectra of SPF and SPLN in buffer and in the presence of lipid vesicles and (A) SPF or (B) SPLN. (—) buffer; (—•—) CL; (– – –) DOPG; (—·—) DOPC: (–××–) PS; (···) *E. coli* lipids. Peptide concentration = 3 μM, lipid concentration, 420 μM.

upon addition of SUV. Emission spectra were recorded 5 min after addition with the excitation monochromator set at 280 nm. The increase in fluorescence intensity was calculated at a fixed wavelength after correction for volume change. Fluorescence titration curves were analyzed according to Bashford et al. (1979) and Surewicz and Epand (1984):

$$\epsilon - 1 = (\epsilon_b - 1) - K_d(\epsilon - 1)/nm$$

where K_d is the dissociation constant of the lipid–peptide complex, m is the lipid concentration, n is the number of binding sites per lipid; ϵ represents I/I_o, the relative change of fluorescence intensity, and ϵ_b is the maximum relative change of fluorescence intensity attainable when all peptide is bound. The parameter K_d/n, obtained as the slope of a plot of $(\epsilon - 1)$ vs $(\epsilon - 1)/m$, provides an indication of the affinity of the ligand to the membrane.

The influence of quenchers on the tryptophan fluorescence of SPF and SPLN was studied by addition of increasing amounts of 4 M KI, 2 M $NaNO_3$, and 3 M acrylamide solution and monitoring quenching of fluorescence. The KI solution contained 1 mM $Na_2S_2O_3$ to prevent I_3^- formation. The data was analyzed according to the Stern–Volmer equation for collisional quenching (Lehrer, 1971): $I_o/I = 1 + K_{sv}[Q]$, where I_o and I are the fluorescence intensities in the absence and presence of quencher, [Q] is the molar concentration of quencher, and K_{sv} is the Stern–Volmer quenching constant.

The ability of SPF and SPLN to cause release of CF was checked by monitoring the fluorescence intensity of CF encapsulated in PS, DOPC, and DOPG vesicles at self-quench concentrations (Weinstein et al., 1977; Blumenthal et al., 1977; Nagaraj et al., 1987). Lipid film (DOPC, PS, or DOPG) was dispersed in 5 mM Hepes, pH 7.4, containing 100 mM CF and sonicated to clarity. Liposomes were separated from nonencapsulatated CF by gel filtration on Sephadex G-75 (elution buffer 5 mM Hepes, pH 7.4/150 mM NaCl, 1 mM EDTA). The excitation monochromator was set at 493 nm, and the emission at 520 nm was continuously monitored after addition of peptide from a stock solution to the lipid vesicles. The fluorescence of CF is enhanced considerably on dilution, and hence the increase in fluorescence is a measure of vesicle permeabilization. Complete release of CF was obtained by addition of Triton X-100 (0.1% v/v). A stock solution of SPF was prepared in methanol. The concentration of methanol was kept below 1% in all experiments. A stock solution of SPLN was made in water. All experiments were carried out at 25 °C. Fluorescence experiments were carried out in a Hitachi 650-10S spectrofluorimeter.

RESULTS

Trp Fluorescence. SPF and SPLN have a single Trp residue whose fluorescence can be exploited for estimating binding to lipid vesicles as well as location in the bilayers, as the fluorescence spectrum of Trp is sensitive to environment. The fluorescence spectra of SPF and SPLN are shown in Figure 2, panels A and B. In buffer, SPF and SPLN show a λ_{max} ~350 nm indicating that Trp is exposed to aqueous environment. Enhancement in intensity and a blue-shift of λ_{max} is observed for SPF and SPLN in the presence of DOPC, PS vesicles, and also those composed of lipids from *E. coli*. The shift in λ_{max} is greater for SPLN. Only a blue-shift without any increase in quantum yield is observed for SPF in the presence of DOPG and CL vesicles (lipids with a glycerol head group and CL occur in *E. coli*). However, SPLN, shows an increase in quantum yield as well as a blue-shift of λ_{max} in the presence of DOPG vesicles. Also, in the presence of CL vesicles, the shift in λ_{max} is considerably more than in the case of SPF. Fluorescence characteristics of Trp of SPLN and SPF were monitored as a function of lipid concentration at a fixed peptide concentration. The data were analyzed as described under Materials and Methods to obtain the affinity

Table I: Binding Parameters of SPLN and SPF Interacting with Lipid Vesicles

lipid	K_d/n (mM) SPLN	SPF
DOPC	8.066	3.204
PS	2.192	1.449
E. coli lipids	1.028	2.025

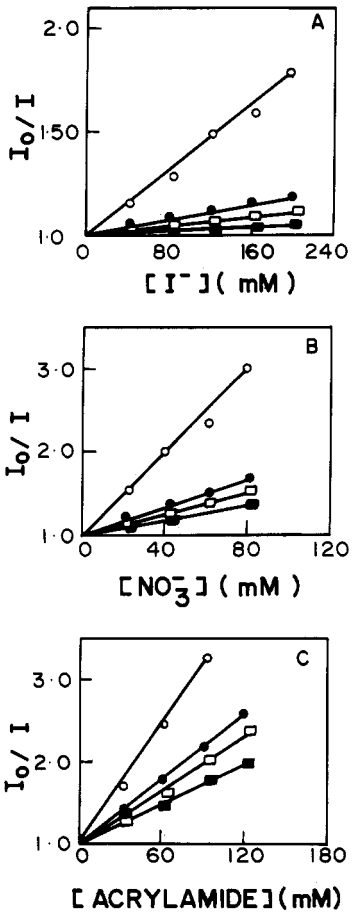

FIGURE 3: Stern–Volmer plots of the quenching of SPF intrinsic fluorescence by (A) iodide, (B) nitrate, and (C) acrylamide, (○) in the absence of lipid and in the presence of (●) DOPC, (□) CL, and (■) DOPG. Initial concentration of peptide = 3 μM and lipid = 480 μM.

parameters, K_d/n, which are summarized in Table I. The values indicate that SPF binds more strongly to PS vesicles as compared to vesicles composed of *E. coli* lipids and DOPC. SPLN binds much more strongly to vesicles composed of *E. coli* lipids as compared to PS or DOPC. Also, the affinity of SPF to DOPC vesicles is more than that of SPLN.

Influence of Aqueous Quenchers. In order to get an insight into the localization of Trp in SPF and SPLN in the lipid bilayer, the effect of various aqueous quenchers on its fluorescence was examined. Stern–Volmer plots of quenching of SPF intrinsic fluorescence in the presence of I^-, NO_3^-, and acrylamide are shown in Figure 3. The results indicate that the fluorophore is almost completely shielded from the aqueous quencher I^- in all the lipid systems. The Stern–Volmer quenching constants are summarized in Table II. The differences in the Stern–Volmer quenching constant K_{sv} suggest a better shielding in DOPG vesicles. The discrimination is less with NO_3^- due to its greater penetrating ability. The K_{sv} values indicate less efficient quenching with acrylamide, which has the ability to penetrate the lipid bilayer.

Table II: Quenching Constants for Free SPLN and SPF in Solution and When Bound to Lipid Vesicles

peptide	lipid	quenching constant (M^{-1}) I^-	NO_3^-	acrylamide
SPLN	none	9.37	48.7	32.6
	DOPC	0.33	8.35	12.5
	DOPG	0.45	7.88	11.0
	CL	0.54	6.78	9.9
SPF	none	4.2	26.4	24.7
	DOPC	0.93	6.38	13.0
	DOPG	0.22	5.55	8.8
	CL	0.56	7.00	12.3

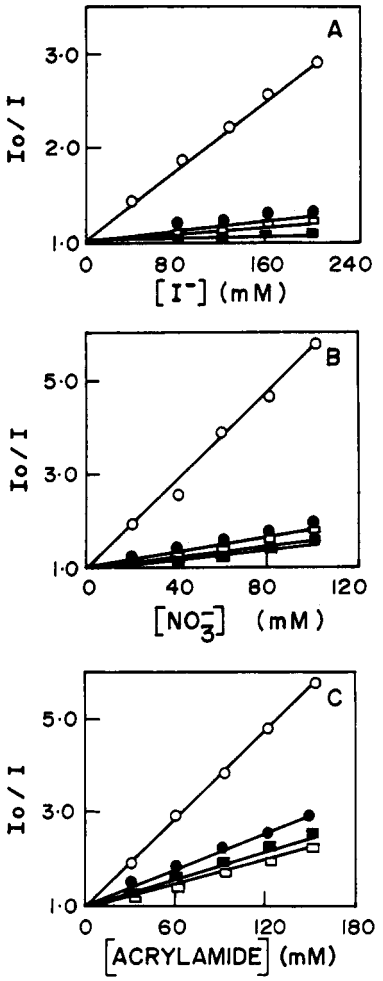

FIGURE 4: Stern–Volmer plots of the quenching of SPLN intrinsic fluorescence by (A) iodide, (B) nitrate, and (C) acrylamide, (○) in the absence of lipid and in the presence of (●) DOPC, (□) CL, and (■) DOPG. Initial concentration of peptide = 3 μM and lipid = 480 μM.

The Stern–Volmer plots for SPLN are presented in Figure 4. Here too, the fluorescence is considerably quenched in the presence of aqueous as well as membrane-penetrating quenchers. The K_{sv} values (summarized in Table II) indicate a similar environment for Trp in all the lipid systems.

Carboxyfluorescein Release. In order to determine whether association of SPF and SPLN with lipid vesicles resulted in alteration of permeability properties, release of entrapped carboxyfluorescein (CF) was monitored. Efflux of CF from DOPC vesicles is shown in Figure 5. An initial rapid release followed by a slow release is observed for both peptides. The extents of CF release, which can be taken as a measure of perturbing ability of the peptides, at different lipid–peptide ratios are comparable. Release of CF from PS vesicles by

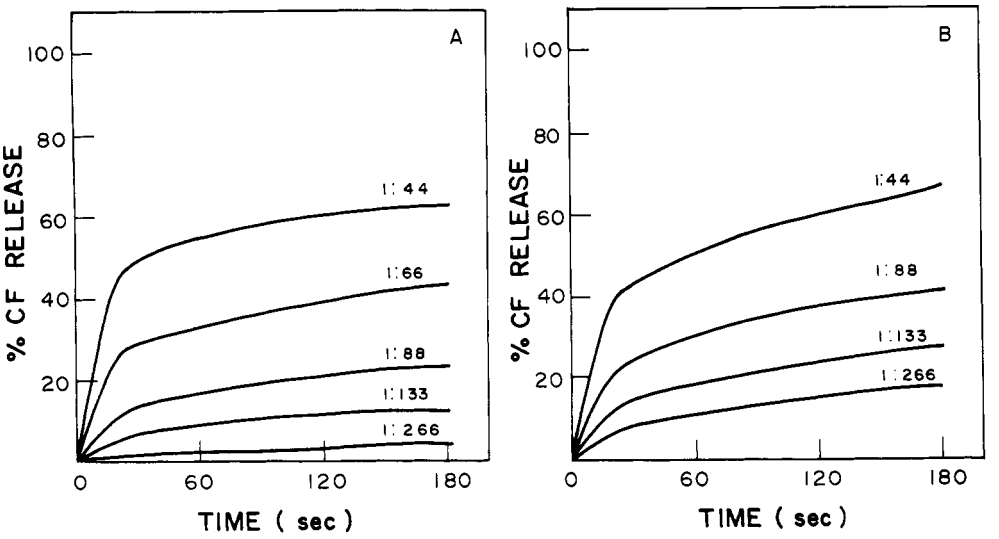

FIGURE 5: Release of CF from DOPC vesicles at different peptide–lipid ratios by (A) SPF and (B) SPLN. Lipid = 400 μM. Release obtained with Triton X-100 was taken as 100%. Peptide–lipid ratios are indicated above each trace.

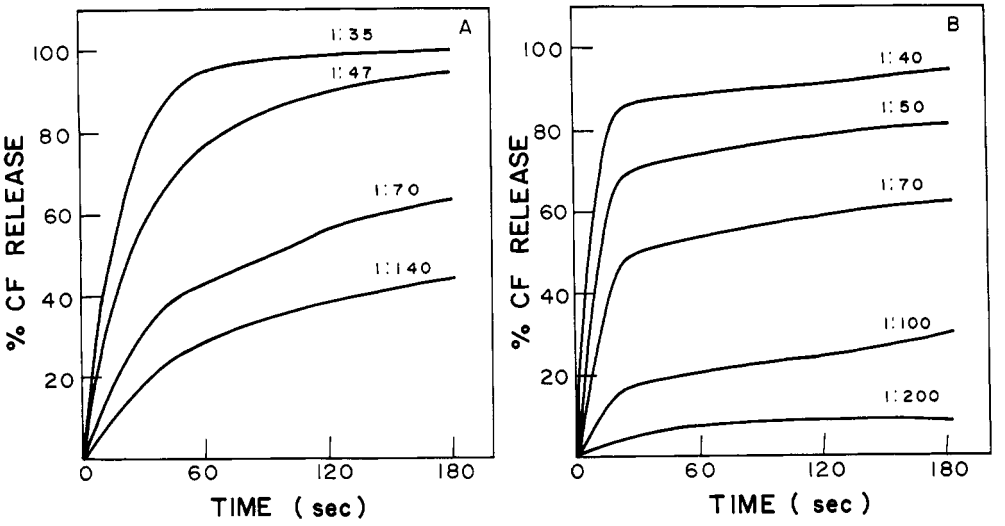

FIGURE 6: Release of CF from PS vesicles at different peptide–lipid ratios by (A) SPF and (B) SPLN. Lipid = 400 μM. Release obtained with Triton X-100 was taken as 100%. Peptide–lipid ratios are indicated above each trace.

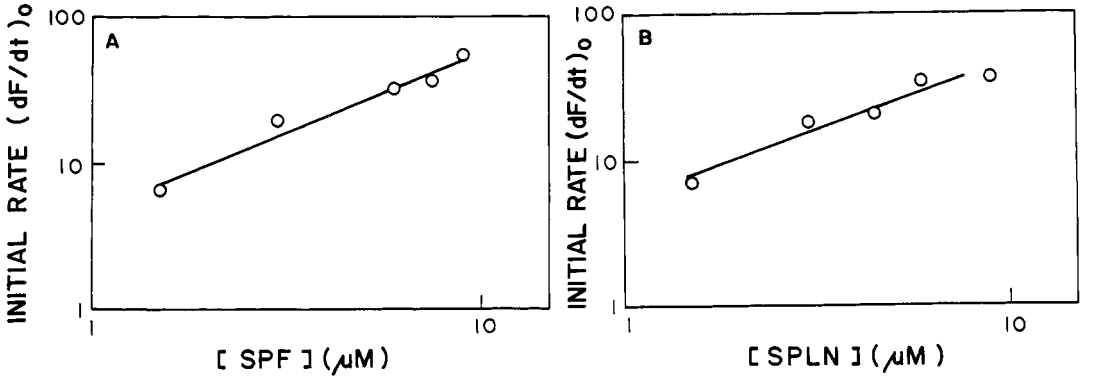

FIGURE 7: Double logarithmic plots of the initial rate of CF release from DOPC vesicles versus peptide concentration. (A) SPF. (B) SPLN. Lipid = 400 μM.

SPF and SPLN is shown in Figure 6. The CF release profiles and the peptide–lipid ratios indicate that both peptides perturb PS vesicles more than PC vesicles. The activity of peptides against CF entrapped in DOPG vesicles was also monitored. Here, SPLN clearly perturbed the DOPG vesicles more than SPF. Plots of log initial rate versus log concentration obtained from CF release profiles from DOPC vesicles are shown Figure 7. A slope 1.0 is obtained from SPF and SPLN. This indicates that release of CF has a first-order dependence on peptide concentration. Similar results were obtained by analysis of CF release profiles from PS and DOPG vesicles.

The CF release profiles at different lipid–peptide ratios, from large unilamellar vesicles made by extrusion of multilamellar vesicles through 0.1-μm filters (Lipex Co., Vancouver, BC) with multiple passes, were very similar to those observed with SUV.

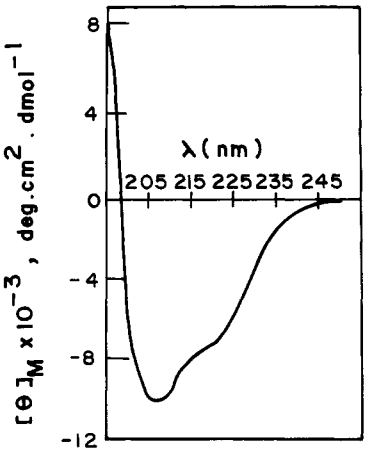

FIGURE 8: Circular dichroism spectrum of SPLN (c = 0.06 mM) in micelles of sodium dodecyl sulfate (30 mM). The spectrum was recorded in a Jobin Yvon dichrograph V spectropolarimeter in the cells of 1-mm path length at 25 °C. $[\theta]_M$ is the mean residue ellipticity.

Circular Dichroism Studies. The CD spectrum of SPLN in micelles of sodium dodecylsulphate is shown in Figure 8. The spectrum is characteristic of peptides in a helical conformation. Although the mean residue ellipticity value at 222 nm is somewhat low, the cross-over at ~200 nm argues against a large percentage of random conformation.

DISCUSSION

SPLN exhibits antibacterial activity against a variety of microorganisms (Reddy & Bhargava, 1979) but does not have hemolytic activity. SPF, a 13-residue peptide corresponding to a region of SPLN considerably more hydrophobic than rest of the peptide, possesses antibacterial as well as hemolytic properties (Sitaram & Nagaraj, 1990). Both SPF and SPLN have the ability to permeabilize the bacterial inner membrane, which results in subsequent cell death (Sitaram et al., 1992). The lysis of erythrocytes by SPF also proceeds through a colloid osmotic lysis process. Studies on the interaction of SPLN with sperm membranes have indicated that the peptide increases the fluidity of sperm plasma and acrosomal membranes (Shivaji, 1986). The interaction of caltrin (a peptide whose sequence is identical to that of SPLN) with lipids has also been implicated in modulation of its calcium-transport regulating activity in bovine spermatozoa (San Agustin & Lardy, 1990). Since the biological properties of these peptides appear to stem from their ability to alter permeability properties of membranes, we have investigated the interaction of both peptides with lipid vesicles with a view to establish their topography in the lipid bilayer and also the consequences of their association with lipid vesicles.

The fluorescence characteristics of Trp in these peptides indicate strong binding to lipid vesicles, like other hydrophobic peptides. SPLN binds more strongly to DOPG vesicles compared to zwitterionic and PS vesicles. Bacterial lipids contain lipids with glycerol as the head group (Wilkinson, 1988). The lower minimal inhibitory concentration against *E. coli* for SPLN as compared to SPF would arise due to a greater affinity of SPLN for bacterial membranes. Localization studies using quenchers indicate that the Trp residues in both the peptides are located away from the head group region and are associated with the hydrophobic core. SPLN has been shown to incorporate into dimyristoylphosphatidylcholine and phosphatidic acid vesicles, and on the basis of quenching experiments with nitroxide probes it has been suggested that Trp is localized near the bilayer surface (Galla et al., 1985). The K_{sv} values for the two peptides suggest a similar location for Trp in the lipid bilayer.

Both peptides cause release of CF from zwitterionic as well as anionic vesicles. Double logarithimic plots of initial rate of CF release versus concentration yield a slope 1 for SPF and SPLN. A higher order of dependence of ~6 has been obtained for the channel-forming peptide alamethicin in similar experiments (Schwarz & Robert 1990). For larger protein toxins like tetanus toxin (Menestrina et al., 1989), *E. coli* hemolysin (Menestrina, 1988), colicin E1 (Kayalar & Duzgunes, 1986), and α-toxin of *Staphylococcus aureus* (Forti & Menestrina, 1989), a slope of 1 has been obtained, and this has been taken as an indication of pore formation of monomers and one to one interaction between toxin molecules and lipid vesicles. While SPF and SPLN do not aggregate in aqueous medium in the range of 1–50 μM, aggregation could conceivably occur at the membrane surface. These performed aggregates at the membrane surface could then form a pore through which CF efflux occurs. In such a situation, a slope of 1 would be obtained from the plots of log initial rate versus log concentrations.

SPF adopts a helical conformation in a hydrophobic environment (Sitaram & Nagaraj, 1990). SPLN also adopts a helical conformation. Although the cross-over occurs at 200 nm, the ellipticity values would suggest a low helical content for SPLN. Conformational analysis based on statistical methods (Chou & Fasman, 1978) yields three short helical segments for SPLN which are shown in Figure 9. Two of the helices would be amphipathic in nature as shown in Figure 9. The lower ellipticity values even in micellar environment could arise due to the absence of a long, continuous helical stretch. However, the cross over at ~200-nm argues against a large extent of random structure. Although a helix of 25 residues would be needed to span the lipid bilayer, several short peptides of 15 residues have pore-forming abilities (Nagaraj et al., 1980; Mathew et al., 1981a,b, 1982; Karle et al., 1986; Mellor & Sansom, 1990; Karle et al., 1991; Balaram et al., 1992; Agarwalla et al., 1992). Assuming a bilayer thickness of 40 Å, short peptides would span only half a bilayer. However, based on a detailed study on the channel forming abilities of mastoparan, a model has been proposed wherein there is distortion of the bilayer at the insertion site (Mellor & Sansom, 1990). The crystal structures of several α-aminoisobutryl-containing peptides indicate helical conformation with a tendency to form channels even in the crystalline state (Karle et al., 1986, 1991; Mellor & Sansom, 1990). Conductance measurements have indicated that these peptides form ion channels in planar bilayers. However, the length of the helix in all these cases would be 25 Å, far less than the theoretical 40 Å needed to span the lipid bilayer. Hence short helical segments of SPLN and SPF could indeed span the lipid bilayer. A possible orientation of SPLN and SPF in membranes is depicted in Figure 10. The monomers shown in the figure could aggregate to form water-filled pores with polar amino acids lining the pore. The amino acids S, R, K, and N in helix 2 (H_2) and K, S, and T in helix 3 (H_3) of SPLN and polar amino acids in SPF can conceivably line the pores. Amino acids like S, K, and T have been found to line the "pores" in several channel proteins (Oiki et al., 1990). The location of Trp, shown in Figure 10 for SPLN and SPF, is consistent with the quenching data presented in Figures 3 and 4.

The model-membrane-perturbing abilities of SPLN and SPF do not seem to be dictated by marked lipid specificity. However, SPLN shows selective antibacterial activity whereas

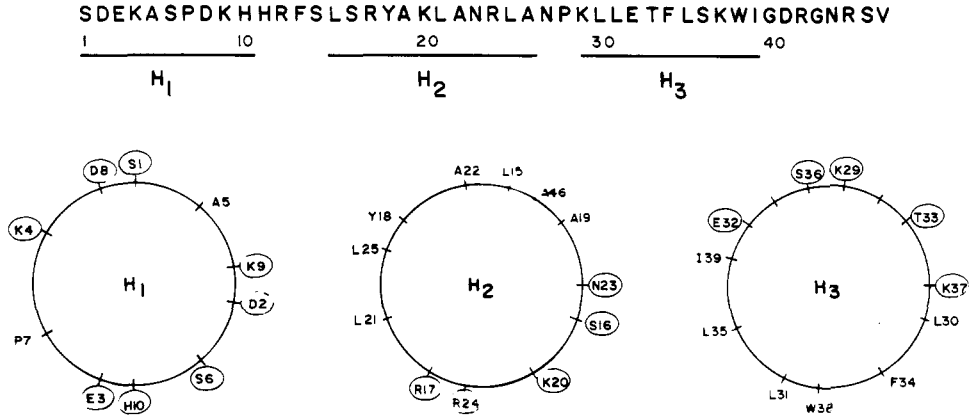

FIGURE 9: Helical regions in SPLN and their projections. H_1, H_2, and H_3 are the helical regions. Polar residues in the helices are circled. Numbers indicate position in the SPLN sequence.

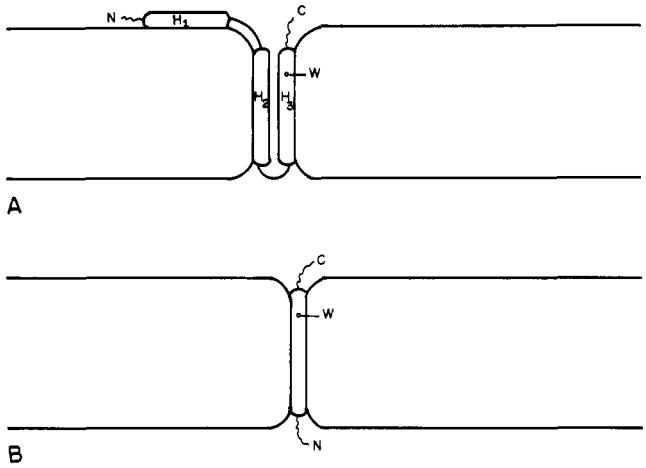

FIGURE 10: Cartoon representation of a model for the association of SPLN and SPF with the lipid bilayer. (A) SPLN. (B) SPF.

SPF exhibits antibacterial as well as hemolytic activities (Sitaram & Najaraj, 1990). This specificity can arise due to differences in the manner in which SPLN and SPF associate with the bacterial and red blood cell surfaces. The outer membrane of Gram-negative bacteria like *E. coli*, which serves as a barrier and thereby provide resistance to most hydrophobic antibiotics (Lutenberg & vanAlphen, 1983), is relatively ineffective in protecting the cell against *cationic* antibiotics (Lutenberg & vanAlphen, 1983; Strom et al., 1977). These molecules bind to the negatively charged lipopolysaccharide molecules located on the exterior of the outer membrane and disrupt the structure, facilitating entry into the cells. The positively charged amino acids in region between S(1) and A(26) and in the C-terminal region (between residues 41 and 47) in SPLN would favor association with the bacterial outer membrane and also cause perturbation and consequently entry into the inner membrane. As the net positive charge in SPLN at neutral pH is more than that of SPF, the former perturbs the bacterial outer membrane more effectively. Although RBCs have zwitterionic lipids on the extracytoplasmic region, it is likely that the positively charged regions of SPLN bind to the negatively charged sialic acid molecules which project out of the RBC surface (Vitala & Jarnefelt, 1985). Since the sialic acid molecules are at a distance from the membrane surface, SPLN cannot reach the lipid bilayer when bound to these sugar molecules. Thus, increased cationicity which is required for enhanced antibacterial activity can be the cause of a peptide lacking hemolytic activity. However, SPF can insert into the RBC membrane in the manner shown in Figure 10B. The biological properties of SPF and SPLN as well as the specific antibacterial activity of SPLN can thus be rationalized in terms of lipid–peptide interactions. Many peptides having antibacterial activity also lyse erythrocytes (Chen et al., 1988; Blondelle & Houghten, 1991). Hence these peptides are of little use for therapeutic purposes. Membrane-active peptides exhibiting specific antibacterial activity would be of considerable interest as resistance against such peptides may not develop easily as against other antibacterial agents. It should be possible to design membrane-active peptides possessing specificity with respect to biological activities such as only antibacterial activity, by judicious positioning of charged amino acids especially those which are cationic. Work on these lines is currently in progress in our laboratory.

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