Anionic Phospholipids Modulate Peptide Insertion into Membranes

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ABSTRACT: While the insertion of a hydrophobic peptide or membrane protein segment into the bilayer can be spontaneous and driven mainly by the hydrophobic effect, anionic lipids, which comprise *ca.* 20% of biological membranes, provide a source of electrostatic attractions for binding of proteins/peptides into membranes. To unravel the interplay of hydrophobicity and electrostatics in the binding of peptides into membranes, we designed peptides *de novo* which possess the typical sequence Lys-Lys-Ala-Ala-Ala-X-Ala-Ala-Ala-Ala-Ala-X-Ala-Ala-Trp-Ala-Ala-X-Ala-Ala-Ala-Lys-Lys-Lys-Lys-amide, where X residues correspond to "guest" residues which encompass a range of hydrophobicity (Leu, Ile, Gly, and Ser). Circular dichroism spectra demonstrated that peptides were partially (40−90%) random in aqueous buffer but were promoted to form 100% α-helical structures by anionic lipid micelles. In neutral lipid micelles, only the relatively hydrophobic peptides (X = L and I) spontaneously adopted the α-helical conformation, but when 25% of negatively charged lipids were mixed in to mimic the content of anionic lipids in biomembranes, the less hydrophobic (X = S and G) peptides then formed α-helical conformations. Consistent with these findings, fluorescence quenching by the aqueous-phase quencher iodide indicated that in anionic (dimyristoylphosphatidylglycerol) vesicles, the peptide Trp residue was buried in the lipid vesicle hydrophobic core, while in neutral (dimyristoylphosphatidylcholine) vesicles, only hydrophobic (X = L and I) peptides were shielded from the aqueous solution. Trp emission spectra of peptides in the presence of phospholipids doxyl-labeled at the 5-, 7-, 10-, 12-, and 16-fatty acid positions implied not only a transbilayer orientation for inserted peptides but also that mixed peptide populations (transbilayer + surface-associated) may arise. Overall results suggest that for hydrophobic peptides with segmental threshold hydrophobicity below that which promotes spontaneous membrane insertion, primary electrostatic attractions provided by anionic phospholipids become essential for peptide binding and insertion to membranes.

Anionic lipids are integral components of biological membranes and are often localized at the site of action in biological processes [*viz.*, signal transduction (Harlan *et al.*, 1995), membrane protein or polypeptide insertion and translocation (Briggs *et al.*, 1986; Tamm, 1991; Schatz & Dobberstein, 1996), and hormone or toxin penetration (Backlund *et al.*, 1994; Lakey *et al.*, 1994)]. Although conclusive experiments within the physiological milieu remain scarce, much circumstantial evidence has accumulated suggesting that negatively charged lipids are crucial: electrostatic interactions between the anionic lipid head groups and positively charged residues of proteins or peptides could attract them from the cytoplasm and the intraorganelle medium to the membrane surface, thus promoting insertion and/or transport [for reviews, see de Vrije *et al.* (1990), de Kruijff (1994), Hirsh *et al.* (1996), and Seelig *et al.* (1996)]. Other reports suggest that association of proteins or peptides with membranes is principally dependent on hydrophobic interactions between the hydrophobic segments of peptides with the hydrocarbon chains of the membranes, while electrostatic interactions may play only a minor role (Law *et al.*, 1986; Cevc *et al.*, 1990; Maitani *et al.*, 1990). In fact, both may be correct, with the relative significance of the two varying from case to case.

Since protein segments targeted for membrane insertion contain a combination of hydrophobic and (often positvely) charged residues, and most biomembranes contain negatively charged lipids (typically ∼20%), it can be expected that hydrophobic interactions and electrostatics will play distinct roles in various peptide−protein/lipid binding processes (Govers-Riemslag *et al.*, 1994; Leenhouts *et al.*, 1995; Snel *et al.*, 1995); most often, association of proteins/peptides with membranes likely involves both. For example, the binding of myristoylated Ala-rich protein kinase C substrate to biological membranes was found to require both hydrophobic insertion of its myristoyl chain into the lipid bilayer and electrostatic interaction of its basic domain with acidic lipids (Kim *et al.*, 1994). In another instance, the insertion of Sec A into phospholipids, which is believed to be important for the translocation of precursor proteins across the inner membrane of *Escherichia coli*, is stimulated by negatively charged lipids, but hydrophobic interactions were also shown to play a significant role (Breukink *et al.*, 1992). In a further instance, studies of the association of human blood coagulation factors VIII, IXa, and X with phospholipid vesicles found that the association of lipids with factor VIII is primarily hydrophobic, while the association of lipids with factor IXa or X is largely due to the electrostatic forces (Atkins & Ganz, 1992).

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Although a variety of approaches have been employed to study the role(s) of hydrophobic and electrostatic interactions in peptide (protein) binding with membranes (de Kroon *et al.*, 1990; Swanson & Roise, 1992; Reynaud *et al.*, 1993; Wimley & White, 1993; Johnson & Cornell, 1994; Wang & Weiner, 1994; Soulages *et al.*, 1995; Heymann *et al.*, 1996), a general scheme for the relative contributions of these two important forces remains to be promulgated. To examine the relevant factors systematically, we here exploit the interaction of model peptides with lysophospholipid micelles and lipid bilayers; peptides were designed to be positively charged and to possess varying degrees of hydrophobicity, while zwitterionic (net neutral) and anionic phospholipids were employed to construct variably charged micelles/vesicles. Circular dichroism measurements were used to detect the influence of phospholipids on the secondary structures of peptides. Fluorescence techniques were employed to study the topology of peptides in lipid bilayers by monitoring the fluorescence of the tryptophan residue built into the hydrophobic core of the model peptides.

MATERIALS AND METHODS

Peptide Synthesis and Purification. Detailed procedures for peptide synthesis, purification, and characterization have been described (Liu *et al.*, 1996). Peptides were synthesized by continuous-flow Fmoc[1] solid phase chemistry and were assembled on the NovaSyn KR 125 resin (Atherton & Sheppard, 1990). C-termini of peptides were aminated after cleavage from the resin. Deprotection and cleavage of the peptides were carried out in a mixture of trifluoroacetic acid/water/anisole/ethanedithiol (95:1:2:2). The crude peptides were desalted on a Sephadex G-10 column eluted using 20 mM ammonium bicarbonate and were then lyophilized. Purification of peptides was carried out on a reverse-phase Vydac-C4 semipreparative HPLC column (10 × 250 mm, 300 Å), using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Purified peptides were characterized by analytical HPLC, amino acid analysis and mass spectrometry. Concentrations of peptides were determined in triplicate, through quantitative amino acid analysis using Ala recovery as the standard. Peptides were stored as solid powders at −20 °C.

Phospholipids. Synthetic lysophosphatidylglycerol (LPG, C14:0), lysophosphatidylcholine (LPC, C14:0), dimyristoylphosphatidylglycerol (DMPG), dipalmitoylphosphatidylglycerol (DPPG), dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), and 1-palmitoyl-2-(*n*-doxylstearoyl)phosphatidylcholines (*n*-doxyl PC, *n* = position of doxyl in the stearoyl chain; here, *n* = 5, 7, 10, 12, and 16) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Lipids can be stored as powders at −20 °C for 12 months. Purity of lipids was routinely determined by thin-layer chromatography [$CHCl_3/CH_3OH/H_2O$ (volume ratio = 65:35:3)]. Electrospray mass spectrometry was used to check the doxyl-labeled specificity and purity of spin-labeled lipids, from which the correct molecular ion peak (MW = 862.20) was found for each lipid.

Sample Preparation. Micellar solutions (10 mM LPG and 10 mM LPC) were prepared by dissolving desired amounts of lipids into 10 mM Tris-HCl, 10 mM NaCl, pH 7.0 buffer, followed by addition of the peptide stock solution. Peptide concentrations for circular dichroism (CD) measurements were adjusted to 30 μM; for fluorescence measurements, the peptide concentration was 4 μM.

Incorporation of the peptides into vesicles was carried out as previously reported [MacDonald & MacDonald, 1975; see also Liu *et al.* (1996)]. A solution of peptide in chloroform/methanol (2:1) was mixed with DMPG or DMPC in chloroform/methanol (2:1) (peptide:lipid = 1:100 mol/mol). The solvent was evaporated under N_2, until a lipid film was obtained at the bottom of the test tube; the film was then further dried under vacuum overnight. The lipid film was hydrated for 30 min in 10 mM Tris-HCl buffer, pH 7.0, with vortexing, with caution taken to ensure that the temperature was maintained at 10 °C above the phase transition temperature of the lipids. The vesicle suspension was sonicated using a bath-type sonicator until the cloudy solution became clear, typically after 40−60 min. During the sonication, the temperature of the water bath was maintained around 30 °C to prevent damage of the lipid vesicles. The resulting small unilamellar vesicles (SUVs) were used directly for spectroscopy measurements.

Circular Dichroism Measurements. All measurements were performed on a Jasco-720 spectropolarimeter using a 1 mm quartz cell at 25 °C. Each spectrum was the average of four scans with background of the buffer subtracted. Peptide concentration was typically 30 μM in aqueous buffer, in various ratios of lipid micellar solutions, or in lipid vesicles. The aqueous buffer was prepared from 10 mM Tris-HCl, 10 mM NaCl, pH 7.0. Freshly prepared lipid samples were used to minimize oxidation.

Fluorescence Spectroscopy. Fluorescence spectra were recorded on a Hitachi F-400 fluorescence spectrophotometer with emission and excitation slit widths of 5 nm. Excitation wavelength was 280 nm. Emission spectra were recorded from 300 to 400 nm. Samples for maximum fluorescence emission wavelength measurements were prepared as follows: the peptide concentration was 4 μM in aqueous buffer, in 0.4 mM lipid micelles, or in 0.4 mM lipid vesicles.

For iodide quenching experiments, samples in 10 mM Tris-HCl, pH 7.0 buffer contained 4 μM peptide, 0.4 mM DMPG, or DMPC lipid vesicles, along with the indicated concentration of potassium iodide (0, 0.02, 0.04, 0.08, 0.10, 0.14, 0.16, and 0.20 M). Potassium chloride, which does not quench fluorescence, was added to bring a total salt concentration to a constant value of 0.20 M for all samples. The spectra were recorded at 37 °C in a 1 cm disposable acrylic cuvette with subtraction of the background of buffer or lipids in the absence of peptide.

For depth quenching measurements, incorporation of peptides into vesicles was performed as described above, except that mixed phospholipids were used (molar ratio of spin-labeled lipid *n*-doxyl PC to DPPG was 1:9). The vesicle suspension was sonicated on ice using a probe sonicator (two successive 30 s bursts) instead of a bath sonicator to shorten the elapsed sample preparation time. For these experiments,

[1] Abbreviations: CD, circular dichroism; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high-performance liquid chromatography; LPG, lysophosphatidylglycerol; LPC, lysophosphatidylcholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol); DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol); DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; *n*-doxyl PC, 1-palmitoyl-2-(*n*-doxylstearoyl) phosphatidylcholine, where *n* = 5, 7, 10, 12, or 16; TLC, thin-layer chromatography.

Table 1: Primary Structures and Hydrophobicities of Model Peptides

peptide[a]	primary structure (1 5 10 15 20 25)	$H(K-D)$[b]	H(GES)[c]
A_2IA_2	KK<u>AAAIAAAAAIAAWAAIAAA</u>KKKK-amide	2.08	−1.85
A_2LA_2	KK<u>AAALAAAAALAAWAALAAA</u>KKKK-amide	1.97	−1.81
A_2GA_2	KK<u>AAAGAAAAAGAAWAAGAAA</u>KKKK-amide	1.31	−1.52
A_2SA_2	KK<u>AAASAAAAASAAWAASAAA</u>KKKK-amide	1.25	−1.46

[a] Single letter codes of amino acids are used. The hydrophobic segment of each peptide is underlined. [b] Average hydrophobicity of the hydrophobic segment of each peptide, calculated by Kyte−Doolittle scale (Kyte & Doolittle, 1982). [c] Average hydrophobicity of the hydrophobic segment of each peptide, calculated by GES scale (Engelman *et al*., 1986).

spectra were recorded at 50 °C to maintain the lipids in the liquid-crystalline state.

RESULTS

Peptide Design. The peptide sequences shown in Table 1 were originally designed as single transbilayer hydrophobic peptide models; the rationale for this *de novo* design has been reported (Liu et al., 1996). The typical sequence of the peptides is Lys-Lys-Ala-Ala-Ala-X-Ala-Ala-Ala-Ala-Ala-X-Ala-Ala-Trp-Ala-Ala-X-Ala-Ala-Ala-Lys-Lys-Lys-Lys-amide. Specific features of this design are (i) a 19-amino acid hydrophobic core, which allows the peptide to attain stable secondary structures upon interactions with lipid bilayers and potentially transverse a lipid bilayer in a single α-helix conformation; (ii) flanking of the hydrophobic core by hydrophilic positively charged Lys residues to enhance water solubility and facilitate workup; and (iii) a Trp residue incorporated into the peptide hydrophobic core to provide an intrinsic probe to monitor the microenvironment of the peptide by fluorescence spectroscopy.

Peptides designed in this study possess three guest X residues in the hydrophobic core, such that the hydrophobicity of the peptide can be adjusted by systematic replacement of the X residues with hydrophobic or hydrophilic amino acid residues. In the present work, guest residues are chosen from generally recognized hydrophobic amino acid residues Ile and Leu and less hydrophobic residues Ser and Gly, thereby creating two *de facto* categories of peptides: the average hydrophobicities of A_2IA_2 and A_2LA_2 are greater than the threshold value which dictates hydrophobic peptide segments to insert and adopt fully α-helical conformations in the membrane environment (Liu *et al.*, 1996), while the average hydrophobicities of A_2SA_2 and A_2GA_2 are lower than this threshold value. The peptides are designated as A_2LA_2 (L = Leu), A_2IA_2 (I = Ile), A_2GA_2 (G = Gly), and A_2SA_2 (S = Ser); this nomenclature employs the standard single letter code for amino acids to reflect the periodic quintet subsequence in their hydrophobic cores. The average hydrophobicity of each peptide hydrophobic core was calculated by the Kyte−Doolittle scale (Kyte & Doolittle, 1982) and the GES scale (Engelman *et al.*, 1986), with the resulting order of peptide hydrophobicities being A_2IA_2 > A_2LA_2 > A_2GA_2 > A_2SA_2 (Table 1).

Interaction of Peptides with Lipid Micelles. To investigate the specificity of interaction of peptides with lipid micelles with respect to lipid head groups, anionic and zwitterionic lysolipids were used. As shown in Figure 1b, CD spectra

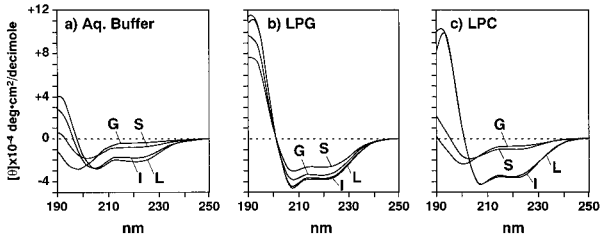

FIGURE 1: CD spectra of model peptides in (a) aqueous buffer, (b) 10 mM LPG micelles, and (c) 10 mM LPC micelles. The spectrum corresponding to each peptide A_2XA_2 (sequences as given in Table 1) is identified as a single letter (X) for clarity. Peptide concentration was 30 μM. Aqueous buffer = 10 mM Tris, 10 mM NaCl, pH = 7.0. See Materials and Methods for details of CD measurements.

Table 2: α-Helical Contents of Model Peptides in Various Media

	α-helical contents[a]			
peptide	aq[b]	LPG[c]	LPC[c]	LPC/LPG (3:1)[c]
A_2IA_2	49	100	100	100
A_2LA_2	59	100	100	100
A_2GA_2	9	94	20	93
A_2SA_2	21	75	28	72

[a] Values are expressed as α-helical percentages. Ellipticity at 222 nm in CD spectra is used as a measure of relative helicity, where $\theta_{222} = -35\,100$ deg cm²/dmol is taken as 100% α-helix (Chen *et al*., 1974). [b] Aq, aqueous buffer = 10 mM Tris-HCl, 10 mM NaCl, pH 7.0. [c] Lipid micelle concentration = 10 mM lysophopholipid(s) in aqueous buffer.

of the peptides in anionic LPG micelles demonstrate that all the peptides (X = I, L, G, and S) were promoted to adopt α-helical conformations, with characteristic double minima at 208 and 222 nm, as compared with the same peptides in aqueous buffer (Figure 1a), where A_2IA_2 and A_2LA_2 peptides were partially helical, while A_2GA_2 and A_2SA_2 were essentially random. The α-helical contents of the peptides in various media were calculated and are listed in Table 2. Peptide helicity in LPG micelles is correlated to its average segmental hydrophobicity, suggesting that the more hydrophobic peptide adopts the more stable α-helical conformation. When zwitterionic LPC micelles were used (Figure 1c), a clear segregation in peptide secondary structure is observed: for the hydrophobic peptides A_2IA_2 and A_2LA_2, there is no significant change of their (fully helical) secondary structures *vs* the peptides in LPG micelles, while for the less hydrophobic peptides A_2GA_2 and A_2SA_2, peptide conformations (largely random) resemble those in aqueous solution.

Mixed lipid micelles were then prepared by using varying molar ratios of zwitterionic LPC and negatively charged LPG (LPC:LPG = 1:3, 1:1 and 3:1, respectively). Peptide secondary structure as α-helix (shown for A_2SA_2 and A_2LA_2 peptides, respectively, in Figure 2, panels a and b) was found to be stabilized when the micelles contained 25% negatively charged lipids to mimic the relative content of anionic lipids in biological membranes. Thus, for hydrophobic peptides A_2IA_2 and A_2LA_2, peptide secondary structure is essentially independent of the head group charge of the lipid micelles, but for less hydrophobic peptides A_2SA_2 and A_2GA_2, anionic lipids play a determining role in stabilizing the peptides' α-helical conformations.

Fluorescence emission of the Trp residue incorporated into the hydrophobic core of each peptide provides another practical route to monitor the binding of peptides with the lipids. In aqueous solution, the Trp fluorescence emission

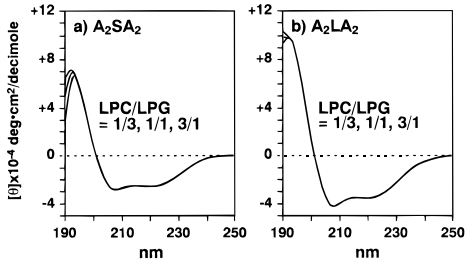

FIGURE 2: CD spectra of peptides (a) A_2SA_2 and (b) A_2LA_2 upon interaction with mixed micelles of LPC/LPG. Mixed micelles are composed of LPC/LPG (3:1, 1:1, and 1:3, respectively), as labeled on the diagram. Note that the three CD curves essentially overlap in panels a and b. In parallel experiments, A_2IA_2 and A_2GA_2 (not shown) displayed similar, overlapping curves. Peptide concentration = 30 μM; mixed micelle concentration = 10 mM in 10 M Tris-HCl, 10 M NaCl, pH 7.0.

Table 3: Tryptophan Fluorescence Emission Maxima (λ_{max}) for Model Peptides in Micelles[a]

	Trp λ_{max} (nm)[b]			
peptide	aq	LPG	LPC	LPC/LPG (3:1)
A_2IA_2	350	335	335	335
A_2LA_2	351	338	339	338
A_2GA_2	351	338	351	338
A_2SA_2	352	340	350	340

[a] Peptide concentration: 4 μM. Micelle concentration: 0.4 mM in 10 mM Tris-HCl, 10 mM NaCl, pH 7.0. [b] Excitation at 280 nm. Wavelength uncertainty estimated as ±1 nm. See Materials and Methods for details of spectroscopic measurements.

maxima of these peptides were around 350 nm; this value shifted to *ca.* 340 nm in LPG micelles, which suggested that the peptides had inserted into the LPG micelles. However, in LPC micelles, a blue shift of the Trp emission maxima was observed for hydrophobic peptides A_2IA_2 and A_2LA_2, but not for the less hydrophobic peptides A_2SA_2 and A_2GA_2 (Table 3). Similar observations for the peptides in mixed micelles of LPG/LPC also suggested that penetration of the Trp residues of the hydrophobic peptides A_2IA_2 and A_2LA_2 into micelles was not affected by lipid composition; for less hydrophobic peptides A_2SA_2 and A_2GA_2, the Trp residues of the peptides were capable of inserting into the micelles efficiently only when there was net negative charge on the micelle surface. These results are in conformity with those from CD measurements.

Interactions of Peptides with Lipid Vesicles: Trp Fluorescence Quenching of Peptides by Iodide. Negatively charged DMPG and DPPG vesicles and zwitterionic DMPC and DPPC vesicles were employed in Trp fluorescence quenching experiments to compare peptide binding capabilities. All four peptides were shown to be fully bound to PG vesicles by the criterion of size-exclusion HPLC chromatography (*viz.*, no free peptide detected). For PC vesicles, A_2LA_2 and A_2IA_2 were fully bound, while free peptide (estimated from peak areas as > 90% of peptide added) was detected for A_2GA_2 and A_2SA_2 (data not shown). An aqueous-phase quencher such as iodide can quench the fluorescence of tryptophan upon collision (Lakowicz, 1983; Eftink, 1991). Figure 3a shows the spectrum of the peptide A_2LA_2 in aqueous buffer. When 0.2 M KCl (which does not quench fluorescence) was replaced by 0.2 M KI, a 70% reduction in the intensity of the fluorescence at 350 nm resulted. In contrast, KI produced only 18% quenching of the fluorescence intensity for peptide A_2LA_2 in DMPG

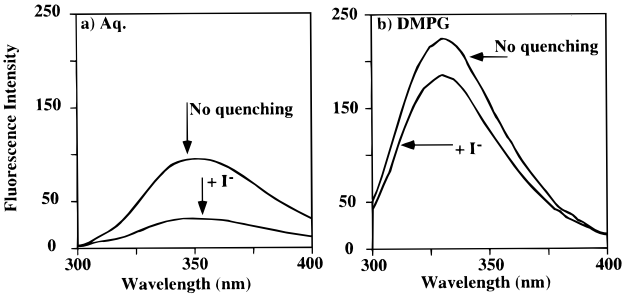

FIGURE 3: Quenching of Trp (residue 15) in peptide A_2LA_2 by aqueous potassium iodide. Excitation wavelength, 280 nm. Peptide concentration = 4 μM. Spectra of unquenched peptide were recorded in 0.2 M KCl, while spectra of quenched peptide were recorded in 0.2 M KI (indicated by arrows in the diagram). (a) Peptide in 10 mM Tris-HCl, pH 7.0. (b) Peptide in the presence of 0.4 mM DMPG vesicles (prepared in 10 mM Tris-HCl, pH 7.0).

vesicles (Figure 3b), suggesting that the presence of the vesicles effectively shields the Trp residue from the aqueous-phase quencher iodide. Significantly, similar results were obtained in the I^- quenching of Trp fluorescence of the other three peptides, indicating that all four peptides interact intimately with DMPG.

Quenching may be examined quantitatively by the Stern−Volmer plot, which relates fluorescence quenching to the concentration of quencher by the equation $F_0/F = K_{sv}[\text{quencher}] + 1$, where F_0 is the peak fluorescence intensity in the absence of quencher, F is the peak fluorescence intensity at a given quencher concentration, and K_{sv} is the Stern−Volmer quenching constant. Figure 4, panels a−c, shows the Stern−Volmer plot of iodide quenching for the peptide in aqueous buffer, in DMPG vesicles, and in DMPC vesicles. The slopes for the hydrophobic peptides A_2LA_2 and A_2IA_2 in the presence of DMPG vesicles and DMPC vesicles are lower than for the peptides in buffer, suggesting that Trp is less accessible in the presence of lipid vesicles. However, comparison of the slopes in DMPG and DMPC indicates that the overall binding of peptides with anionic lipid vesicles is tighter than that with zwitterionic lipid vesicles. The similar slopes for less hydrophobic peptides A_2SA_2 and A_2GA_2 in buffer and in the presence of DMPC vesicles demonstrate that in each of these cases, Trp residues remain in an aqueous environment despite addition of DMPC vesicles. The relatively lower slopes for all four peptides in DMPG vesicles suggest that the Trp residues in these peptides are buried in DMPG vesicles to varying extents, with A_2SA_2 and A_2GA_2 not as tightly associated as the A_2LA_2 and A_2IA_2.

Charged quenchers will be bound to the head groups of lipid vesicles with opposite charge (Griezer & Tausch-Treml, 1980) but repelled from head groups of like charge (Hautula et al, 1973). To avoid any artifacts that might be caused by using iodide as the only quencher, similar experiments were performed using the cation cesium as the aqueous quencher. Although cesium is a weaker quencher, as reflected by its lower K_{sv}, parallel trends to results with iodide experiments were obtained in each case (Stern−Volmer plots not shown).

Orientation of Peptides in Lipid Bilayers. From the above experiments, we know that the peptides have been successfully incorporated into the anionic lipid vesicles. However, due to the Lys residue content at both N- and C-termini, plausible orientations of the peptides include both parallel

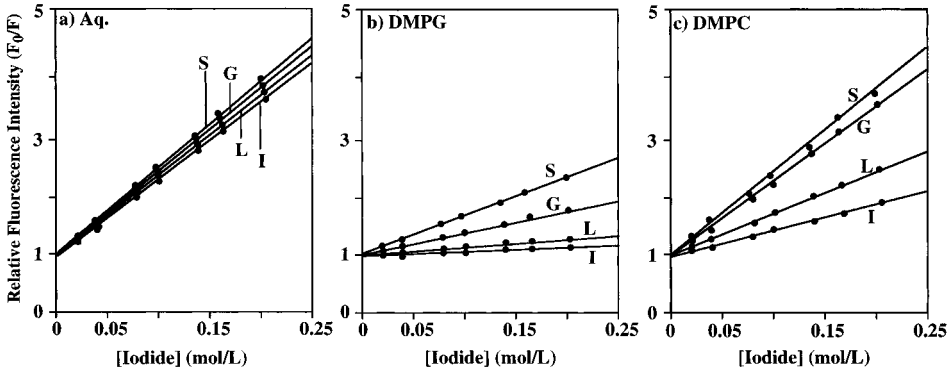

FIGURE 4: Stern−Volmer plot for iodide quenching of peptide tryptophan fluorescence in (a) aqueous buffer, (b) DMPG vesicles, and (c) DMPC vesicles. Peptides are indicated in the diagram as S, G, L, and I for clarity. Peptide concentration = 4 μM. Iodide concentration is given on the x-axis. Salt concentration was maintained in all samples at 0.2 M by addition of KCl. Aqueous buffer was 10 mM Tris-HCl, pH 7.0; DMPG and DMPC vesicles were 0.4 mM in the aqueous buffer.

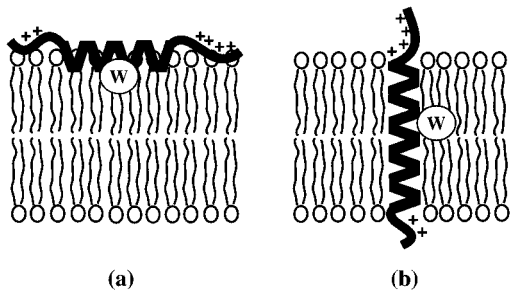

FIGURE 5: Schematic representation of proposed orientations for peptide−lipid interaction: (a) surface-associated peptide; (b) bilayer-spanning (transmembrane) peptide. W = location of Trp residue 15 in the peptide sequence.

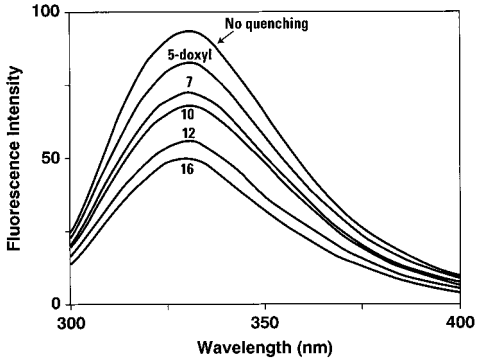

FIGURE 6: Fluorescence quenching shown for peptide A_2IA_2 in DPPG SUVs containing *n*-doxyl spin-labeled PC lipids (molar ratio 9:1 DPPG/doxyl-labeled PC), n = 5, 7, 10, 12, and 16). The peptide was 4 μM in 0.4 mM SUVs. SUVs containing DPPG/DPPC (9:1) were used as unquenched control. Spectra are identified in the diagram by the position of the doxyl label in each experiment. Data for all four peptides are summarized in Table 4.

or perpendicular dispositions with respect to the surface of the lipid bilayer (Figure 5). Taking advantage of the single Trp incorporated into the hydrophobic core of each peptide, the depth of this fluorophore can be detected by spin-labeled lipids, and accordingly the two possible orientations of peptides distinguished. The accuracy of spin-labeled lipids as depth-dependent probes has been extensively demonstrated (Bolen & Holloway, 1990; Chattopadhyay & London, 1987; London & Feigenson, 1981; Markello *et al.*, 1985). The method rests upon the facts that (i) Trp fluorescence within a membrane can be quenched by doxyl stearate upon contact within *ca.* 5 Å and (ii) quenching within the bilayer is primarily static rather than collisional with a distance dependence, so that the highest quenching efficiency should be observed when the doxyl moiety is closest to the Trp fluorophore [reviews: Blatt and Sawyer (1985) and London (1982)].

The comparison of the Trp emission of peptide A_2IA_2 in the presence of SUVs composed of DPPG/n-doxyl PC (molar ratio is 9:1; n = 5, 7, 10, 12, and 16) with that in the presence of DPPG SUVs containing 10% n-doxyl PC is shown in Figure 6. As can be seen, Trp is quenched significantly by the deeply located quenchers (12- or 16-position), but less efficiently by the shallow quenchers (5- or 7-position). Considering the estimated depth of variously positioned doxyl moieties with respect to the center of the lipid bilayer (Chattopadhyay & London, 1987), this result suggests that the Trp residue in A_2IA_2 is located close to the center of the bilayer as this peptide adopts a transbilayer configuration. Nevertheless, there is also significant quenching by doxyl stearates at the 5- and 7-positions, which are a considerable distance (*ca.* 10 Å from the bilayer center) from a Trp residue ostensibly located near the center of the bilayer. Combining these results with those from iodide quenching experiments, an initial interpretation is that not all of the peptide is bound within the lipid bilayer in a transbilayer configuration, but that a mixed population of peptide configurations (transbilayer peptide + surface-associated peptide) may exist. The results of fluorescence quenching measurements by doxyl-labeled lipids on all four peptides are quantitated in Table 4.

Table 4: Relative Fluorescence Intensity of Peptides in Doxyl Spin-Labeled Vesicles[a]

lipid	relative fluorescence intensity of peptide			
	A_2IA_2	A_2LA_2	A_2GA_2	A_2SA_2
DPPG/DPPC	100	100	100	100
DPPG/5-doxyl PC	89 ± 2	96 ± 2	92 ± 5	87 ± 3
DPPG/7-doxyl PC	78 ± 3	93 ± 4	88 ± 3	85 ± 5
DPPG/10-doxyl PC	73 ± 2	75 ± 2	74 ± 2	82 ± 5
DPPG/12-doxyl PC	60 ± 1	60 ± 1	60 ± 3	63 ± 1
DPPG/16-doxyl PC	52 ± 2	56 ± 4	58 ± 4	59 ± 2

[a] Fluorescence intensity measured at emission maximum wavelength = 330 nm. Excitation wavelength = 280 nm. Temperature = 45 ± 1 °C. Peptide concentration = 4 μM, in samples containing 0.4 mM DPPG/DPPC (9:1) or DPPG/*n*-doxyl PC (9:1) in 10 mM NaCl, 10 mM Tris buffer, pH = 7.0. The fluorescence intensity is reported as a percentage of the fluorescence intensity in DPPG/DPPC, which was normalized to 100.

DISCUSSION

Synthetic membrane-interactive peptides provide a systematic approach toward unraveling the interplay of hydrophobic and electrostatic interactions which describe peptide−membrane association. Results of the spectroscopic experiments described above may be summarized as follows: (1) the hydrophobic peptides A_2IA_2 and A_2LA_2 bind and insert into lipid micelles or vesicles predominantly *via* hydrophobic interactions between the lipid fatty acyl chains and the peptide hydrophobic segment; (2) the less hydrophobic peptides A_2SA_2 and A_2GA_2 can bind phospholipid micelles or vesicles only by relying on *both* electrostatic and hydrophobic interactions. In the latter case, electrostatics between the anionic lipid head groups and the peptides' Lys residues are indispensable for driving the peptides to the membrane surface to achieve primary binding; subsequent hydrophobic interaction with the lipid fatty acyl chains then stabilizes the interaction and effects peptide insertion.

Since peptides becoming lipid-bound will tend to undergo a transition from largely random to predominantly helical conformations, qualitative correlations between peptide average hydrophobicity and propensity for lipid interactions can be obtained from data in Figure 1. Assuming the Chen *et al*. formula (1974) is applicable in the present experiments, the hydrophobic peptides A_2IA_2 and A_2LA_2 form fully α-helical secondary structure in LPG micelles, while the less hydrophobic peptides A_2SA_2 and A_2GA_2 form only marginally less helical conformation. The order of the α-helical content of these peptides, $A_2IA_2 \sim A_2LA_2 > A_2GA_2 > A_2SA_2$ (Figure 1b), is well-correlated with the order of their average segmental hydrophobicity. In contrast, in the presence of zwitterionic LPC micelles at neutral pH, it is clear that hydrophobicity alone is sufficient to convert only A_2IA_2 and A_2LA_2 to essentially fully α-helical conformations, as the less hydrophobic peptides A_2SA_2 and A_2GA_2 display little or no binding to LPC micelles. In addition, in LPG micelles, the blue shift of Trp fluorescence occurs for all peptides (Table 3) while in LPC micelles, the blue shift was only observed for the hydrophobic peptides A_2IA_2 and A_2LA_2.

The heavy atom aqueous quencher iodide, which is not expected to partition readily into the fatty acyl chain region of the bilayer (Johnson & Cornell, 1994), was used to diagnose the positions of peptide Trp residues when bound into lipid vesicles. Iodide quenching abilities vary among aqueous buffer, DMPG vesicles, and DMPC vesicles (Figures 3 and 4), with the Trp residue generally least accessible to the buffer for peptides incorporated into DMPG vesicles. However, that Trp never becomes completely inaccessible to iodide is shown by the fact that Trp quenching by iodide remained a function of peptide hydrophobicity. In this context, the quenching experiments with various doxyl spin-labeled [DPPC/DPPG (1: 9)] lipid preparations suggested that both incorporated (transbilayer) peptide and surface-associated peptide populations exist (Figure 5). The fact that all four peptides behaved similarly in the doxyl quenching experiments, but display a 25% range in helicity in CD spectra, suggests that likely, the helical content of the surface-bound population of the A_2SA_2 and A_2GA_2 may be frayed due the greater conformational flexibility of more aqueous-based (less penetrated) structures. The observation that doxyl-mediated quenching for the A_2LA_2 peptide is relatively minimal in the region between the membrane surface through the 7-doxyl position may suggest that, of the four peptides examined, the Leu compound is the most uniquely oriented with respect to transmembrane insertion, possibly because of its minimal impact on bilayer packing.

The present results may have broad implications in processes such as protein translocation, including import and export [see reviews: Görlich and Mattaj (1996) and Schatz and Dobberstein (1996)]. Previous studies (von Heijne, 1986; Roise, 1992) have suggested that the binding of signal sequences to membranes may constitute a step prior to their recognition by receptors or translocators. It is interesting to note that export signal sequences are hydrophobic, while import signal sequences are relatively more hydrophilic; in addition, mitochondrial import signal sequences are rich in basic amino acids (Roise & Schatz, 1988; Thornton *et al.*, 1993; Hammen *et al.*, 1994). As well, the distribution of negatively charged lipids in the mitochondrial membrane is asymmetric: more cardiolipin (negatively charged) has been found in the outer membrane than in the inner membrane (Hovius *et al.*, 1993). On the basis of this difference, it is reasonable to postulate that electrostatic interactions of mitochondrial import signal sequences with the outer mitochondrial membrane are essential for eliciting function in the less hydrophobic signal sequences.

The molecular events which guide peptide/protein folding at the membrane surface, including α-helix formation and subsequent helix−helix association (oligomerization) remain to be characterized precisely (Wimley & White, 1996; Deber & Goto, 1996; Engelman, 1996). Our finding that peptides of relatively modest hydrophobicity can employ membrane surfaces rich in anionic lipid as a kind of staging area prior to transmembrane insertion allows the speculation that removal of aqueous-based peptide into a microenvironment of reduced dielectric character promotes the replacement of extant (water-solvated) peptide bonds by their intramolecularly H-bonded forms. Such folding of peptide or protein segments into full helices, with concomitant exposure of hydrophobic side chains to lipid, may be a necessary condition for initiation of insertion.

Thus, hydrophobic peptides, whose segmental threshold hydrophobicity allows direct insertion (Liu *et al*., 1996), bind lipids through a one-step concerted mechanism, in which the hydrophobic interaction between the lipid fatty acyl chains and the peptide hydrophobic segments plays the dominant role. For peptides with hydrophobicity below this threshold, insertion into phospholipid bilayers may be viewed, in effect, as a two-step process, in which primary electrostatic attraction is essential for binding and subsequent insertion. It remains of interest to investigate whether net positive charge on the interacting peptide is prerequisite or whether non-charged polar amino acid side chains (Ser, Thr, Asn, etc.) can fulfill the attractive role. Nevertheless, our overall results suggest that there may be a distinction between those membrane-targeted peptides and membrane protein segments of high intrinsic hydrophobic character which can spontaneously insert into biological membranes (Deber & Goto, 1996) *vs* those of mixed hydrophobicity/hydrophilicity whose biological function is modulated specifically by the preponderance of anionic phospholipids in biomembranes.

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