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Mixtures of a Series of Homologous Hydrophobic Peptides with Lipid Bilayers: A Simple Model System for Examining the Protein–Lipid Interface†

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ABSTRACT: The interactions of several members of a homologous series of peptides with the phospholipid bilayer have been examined by using fluorescence and deuterium NMR spectroscopy, differential scanning calorimetry, and measurements of water-to-bilayer partition coefficients. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) bilayers and tripeptides of the form Ala-X-Ala-*O-tert*-butyl are used as a model system to probe the influence of amino acid side-chain substitution on the insertion of peptides into membranes and the behavior of peptide/bilayer mixtures. Tripeptides with X = Gly, Ala, Phe, and Trp have been examined. All of the tripeptides are water soluble, and all partition into DMPC bilayer vesicles to some extent. The Gly-containing peptide is the least soluble and the Trp-containing peptide the most soluble in the bilayer. The extent of perturbation of the bilayer structure induced by the peptides parallels their bilayer solubility: the Gly and Ala peptides act as simple impurities while peptides containing bulky aromatic rings cause a phase separation. Changes in the fluorescence properties of the Trp analogue upon incorporation into the bilayer indicate that the Trp side chain is probably immersed in the hydrocarbon region of the bilayer. Peptides of this form should serve as easily modifiable model systems with which to examine details of how the bilayer environment affects peptide conformation, as well as how hydrophobic peptides affect the bilayer structure.

Most physical models for the insertion and translocation of proteins and peptides into and across membranes invoke relatively simple partitioning processes that rely upon knowing the free energy of transfer of the hydrophobic portions of proteins from water into the lipid bilayer (Engelman & Steitz, 1981; Jahnig, 1983; Sabitini et al., 1982; von Heijne & Blomberg, 1979; Wickner, 1979). Thus, it is of fundamental importance to understand how the hydrophobic portions of proteins interact with their surroundings. Almost all analyses of the protein insertion/translocation problem make four assumptions: (1) The interior of the bilayer can be treated as a simple bulk hydrophobic phase. (2) Partitioning data for the transfer of hydrophobic amino acid side chains from water to bulk organic phases provide an adequate quantitative basis for the partitioning process. (3) All membrane-spanning proteins are comprised largely of either α or 3_{10} helixes. (4) Protein/water interfacial area analyses which are useful for examining globular proteins in water (Chothia, 1976; Richards, 1977; Guy, 1985) are equally applicable to membrane proteins. These assumptions are not necessarily unreasonable, and some parts of the analyses based upon them are compelling. On the other hand, direct experimental data adequate for the evaluation of these assumptions are lacking. The complexity of intact biological membranes makes it difficult to use them for this purpose. One must therefore turn to simpler systems to examine questions of the solubility of portions of proteins (i.e., peptides) in the bilayer, lipid perturbation induced by the incorporation of peptides, location of the peptides in the bilayer, peptide conformational and motional changes induced upon transfer from H_2O to the bilayer, and the effects of changes in the primary structure of the peptide.

Several model systems aimed at elucidating one or more of these properties are being investigated in various laboratories. Davis and co-workers have employed ^2H NMR and other techniques to examine leucine oligomers that span the bilayer (Huschilt, Hodges, & Davis, 1985; Davis et al., 1983).

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Gierasch and co-workers have been examining the implications of relatively minor sequence alterations on the conformation and membrane binding properties of the signal sequence of the LamB protein (Briggs & Gierasch, 1984; Briggs et al., 1985). Amphipathic α helixes represent a qualitatively different class of membrane-associated peptides which Kaiser and co-workers are studying in some detail (Moe & Kaiser, 1985; Kaiser & Kezdy, 1984). Several small to medium size naturally occurring membrane proteins are also being employed as model systems (Dettman et al., 1984; Wilson & Dahlquist, 1985; Braun et al., 1983; Feigenson & Meers, 1980; Weinstein et al., 1980; Eisenberg, 1984).

Work employing short hydrophobic peptides (three to six residues) has shown that manipulation of the amino- and carboxyl-protecting groups can alter the solubility properties of the peptide (Wallace & Blout, 1979; Sugihara et al., 1982). With these results in mind we have been led to develop a class of peptides that can be used to resolve the contributions of single amino acid residues in lipid–protein interactions. These peptides are three residues long with a free amino terminus and the carboxyl end block with a *tert*-butyl ester:

Ala-X-Ala-*O-tert*-butyl

The charged amine serves to anchor that end of the peptide at the lipid/water interface, while the bulky hydrophobic *tert*-butyl group serves to increase peptide solubility in nonaqueous media. Alanine was chosen to be the initial and final residues because it is relatively small and hydrophobic; thus, they will enhance lipid solubility without overwhelming the central residue. Tripeptides are a natural starting point since they are the simplest moieties in which there exists an amino acid residue in a more or less natural setting, i.e., with the peptide bonds on both of its ends. We report here a study of model lipid membranes with admixtures of members of this homologous series of peptides where X is glycine, alanine, phenylalanine, or tryptophan. Buffer to 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)[1] bilayer partition coefficients of the peptides, the thermodynamic phase behavior of the lipid/peptide mixtures, changes in the optical properties of the X = Trp peptide upon partitioning into the bilayer, and ^{2}H NMR spectra of the lipids in lipid/peptide mixtures are presented. We find that even these relatively simple peptides interact strongly with the bilayer and that the characteristics of the interactions are strong functions of the identity of the central residue.

Experimental Procedures

Peptide Synthesis. Peptides of the form Ala-X-Ala-*O-tert*-butyl were synthesized by coupling Ala-*O-tert*-butyl and Z-Ala-X (Vega Biochemicals, Tucson, AZ) and subsequent clipping of the Z protecting group. Coupling was performed with dicyclohexylcarbodiimide via standard methods (Rich & Singh, 1979; Bodanszky et al., 1976; Stewart & Young, 1984). The Z group was then cleaved by hydrogenation over Pd/C catalyst at atmospheric pressure. The product was purified by preparative thin-layer chromatography on silica gel 60 (Whatmann, 20 × 20 × 0.05 cm plates). Purity of the final product was checked by TLC in three solvent systems: 1-butanol–H_2O–acetic acid (8:2:1), chloroform–methanol–H_2O (65:25:5), and ethyl acetate–hexane–acetic acid (20:10:1). Visualization was accomplished with ninhydrin, I_2, and UV absorption on fluorescent plates (silica gel 60 F_{254}).

Lipids. Unlabeled DMPC was purchased from Avanti Polar Lipids (Birmingham, AL). Acyl chain perdeuterated DMPC was purchased from Cambridge Isotopes, Inc. (Cambridge, MA). Tritiated DMPC was purchased from New England Nuclear (Boston, MA).

Vesicle Preparation. Phospholipid vesicles used in the hygroscopic desorption and fluorescence titration studies were prepared by the method of Huang and Thompson (1974). Briefly, lipid suspended in 50 mM phosphate buffer (pH 7.0) was sonicated to clarity (15–30 min) under argon atmosphere with a microtip probe attached to a 250-W Sonics & Materials (Danbury, CT) sonicator. Temperature was maintained at 10–15 °C above the lipid phase transition throughout the sonication. The sample was then centrifuged and decanted to remove titanium particles and large vesicles. Vesicle samples were used within 12 h of their production and maintained above the lipid phase transition temperature.

Peptide/lipid vesicle mixtures were made by combining stock solutions of the peptide and vesicles, diluting to the appropriate volume with buffer, and then bath sonicating under nitrogen for $^1/_2$ h at 33 °C immediately prior to filtration or recording of the fluorescence spectrum.

Hygroscopic Desorption. Bilayer/water partition coefficients were measured by the hygroscopic desorption method (Pjura et al., 1984; Conrad & Singer, 1981). Three filters were mounted in a 47-mm Swin-Lok Holder (Nuclepore Corp.). The top filter was polycarbonate with a nominal pore size of 0.03 μm (Nuclepore Corp. 111102). The middle filter was a Schleicher & Schuell 1-HV inert glass filter, while the bottom filter was a Schleicher & Schuell 470 cellulose pad. The polycarbonate filter was conditioned by washing in 10% acetic acid for about 30 min. The filter was then thoroughly rinsed with deionized H_2O, dried, and used immediately. Treatment of the polycarbonate filter with surfactant [soaking in poly(vinylpyrrolidine)] did not materially affect the filtration times or retention of the vesicle mixtures; thus, this treatment was not routinely incorporated into our experimental protocol.

Peptide/lipid vesicle samples (0.2–0.5 mL, 1–5 mM lipid, 1–5 mM peptide) were filtered for 5–20 min under vacuum. The more concentrated samples took longer to filter, but no dependence of K_p on concentration was observed. The sample, filtering apparatus, and immediate environs were held at 33 °C before and during the filtration. The lipid/peptide mixture was extracted from the filter by washing in 4 mL of 2-propanol. The number of moles of peptide in the prefiltrate sample (C_T) and in the sample remaining on the filter (C_F) was determined by fluorescamine assay (Chen et al., 1978). The radioactivity of the lipid in the prefiltrate sample and in the sample remaining on the filter was used to assay lipid content. Nonspecifically bound peptide was measured in each experiment by running a parallel sample without lipid. Typically, more than 95% of the lipid was retained on the filter, and nonspecifically bound peptide amounted to less than 10% of the total. Values of C_F used in the calculation of K_p were corrected for nonspecifically bound peptide.

The bilayer/water partition coefficient (K_p) was evaluated as the mole fraction of peptide associated with the bilayer divided by the mole fraction free to pass through the filter:

$$K_p = \frac{C_F/(C_F + L)}{C_S/(C_S + W)} \quad (1)$$

where C_S ($=C_T - C_F$) is the number of moles of peptide in the supernatant, L is the number of moles of lipid retained, and

[1] Abbreviations: NMR, nuclear magnetic resonance; $\Delta\nu_q$, deuterium residual quadrupole splitting; DSC, differential scanning calorimetry; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; Z, benzyloxycarbonyl group; A, alanyl; G, glycyl; F, phenylalanyl; K, lysyl; L, leucyl; W, tryptophan.

W is the number of moles of water.

Fluorescence Titration. Fluorescence spectra were measured on a Spex Fluorolog spectrofluorometer (Model 1902) interfaced to a Ditigal Equipment Corp. LSI-11 microcomputer where the spectra were stored and evaluated. Excitation and emission slits were set at 5 nm. The excitation wavelength was 280 nm, scan limits were 290 and 530 nm, and scan speed was 0.5 nm/s. All spectra were measured in the ratio mode by using 10-mm path-length cuvettes thermostated at 33 °C.

The change in the integrated intensity of the Trp emission peak (F) as a function of lipid concentration was used to calculate K_p (Surewicz & Epand, 1984; Bashford et al., 1979). In these calculations R, the enhancement factor, is defined as

$$R = F/F_0 \tag{2}$$

where F_0 is the integrated intensity in the absence of lipid. Following Bashford et al. (1979)

$$\frac{1}{R-1} = \frac{[H_2O]}{(R_m - 1)K_p}\frac{1}{[\text{lipid}]} + \frac{1}{R_m - 1} \tag{3}$$

Linear least-squares analysis of the data plotted in this double-reciprocal fashion yields K_p as defined in eq 1 and R_m, the maximal enhancement factor. Peptide concentration was fixed at 20 μM while the lipid concentration was varied from 0.5 to 5 mM.

Calorimetry. All calorimetric work was performed on a Perkin-Elmer DSC 2B interfaced to a Digital Equipment Corp. LSI-11 microcomputer. DSC samples were prepared by co-dissolving the appropriate amounts of lipid and peptide in chloroform, evaporating the solvent under a stream of dry nitrogen, and then further drying in vacuo overnight. One to three milligrams of the dry sample was weighted into the DSC sample pan, an equal (or somewhat greater) mass of buffer was added, and then the pan was sealed. The sample was then mixed in the calorimeter by cycling the temperature from 275 to 310 K. Typically, no change was observed in the thermogram after the third cycle, although we routinely subjected each sample to seven cycles before recording the thermogram. All thermograms were recorded at a scan speed of 1.25 K/min.

Deuterium NMR. ^{2}H NMR spectra were obtained at the NSF Southern California Regional NMR Center on a Bruker WM-500 spectrometer in the Fourier transform mode at 76.8 MHz (H_0 = 11.7 T). The quadrupole echo technique (Davis et al., 1976) was employed with a τ_{echo} = 40 μs and a 90° pulse of 4–6 μs. The pulse repetition rate was 0.8 s. The data acquisition rate was 166666 Hz. An exponential multiplication factor of 40 Hz was applied to all spectra. Samples were prepared in essentially the same manner as described for the DSC experiments, except then a total mass of approximately 10 mg of dry lipid/peptide mixture and 500 mg of deuterium-depleted water (Aldrich) were sealed in the NMR tube, and the tube was agitated and equilibrated at 30 °C for 18 h before spectra were recorded.

Results

Hygroscopic Desorption. A number of alternative definitions of the water-to-bilyaer partition coefficients (K_p) are available (Katz & Diamond, 1974a; Pjura et al., 1984; Nozaki & Tanford, 1971), but the definition in terms of mole fractions has the advantage of coupling directly measured quantities (see eq 3) with thermodynamic quantities of interest [e.g., the free energy of transfer, $\Delta G_{transfer} = kT \ln (K_p)$] without intervening assumptions, e.g., no molecular volumes need be assumed (Pjura et al., 1984). K_p is defined as

$$K_p = N_{bilayer}/N_{aqueous} \tag{4}$$

Table I: Partition Coefficients of Amphipaths between Lipid Bilayer Vesicles and Buffer[a]

amphipath	lipid	$K_p \times 10^{-3}$
A-G-A-*O-tert*-butyl	DMPC	0.149 ± 0.007 (4)
A-A-A-*O-tert*-butyl	DMPC	0.176 ± 0.006 (6)
A-F-A-*O-tert*-butyl	DMPC	1.03 ± 0.015 (9)
A-W-A-*O-tert*-butyl	DMPC	3.96 ± 0.04 (6)
(fluorescence titration)		5.5 ± 1[b]
1-butanol	DMPC	0.131[c]
cyclohexanol	DMPC	0.333[c]
benzyl alcohol	DMPC	0.524[c]
N-t-Boc-A-W-M-F-D-NH_2	DMPC	13.3[d]
N-t-Boc-A-W-M-G-F-NH_2	DMPC	92.6[d]
palmitic acid	EYL	350 ± 150[e]

[a] Partition coefficients measured in this study were determined by the hygroscopic desorption method and, in addition, by the fluorescence titration method for the Trp-containing peptide. Temperature = 33 °C. Values are given as mean ± SD with the number of experiments in parentheses. [b] Standard deviation estimated from errors in least-squares slope and intercept. Most of the error is associated with the determination of the intercept. [c] Data from Katz and Diamond (1974b) converted to K_p as defined under Results. Temperature = 40 °C. [d] Data from Surewicz and Epand (1984) converted to K_p as defined under Results. Temperature = 40 °C. [e] Data from P jura et al. (1984) converted to K_p as defined under Results.

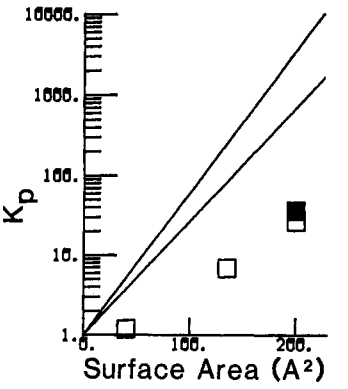

FIGURE 1: Water–bilayer partition coefficients (scaled such that K_p for the Gly peptide is unity) for the tripeptides plotted vs. the accessible surface area of the central amino acid residue (Chothia, 1976) where the Gly area has been subtracted from each. The surface areas increase in the order Gly < Ala < Phe < Trp. Open squares represent the hygroscopic desorption determinations; the closed square is K_p for the Trp peptide determined via the fluorescence titration method. The lines are calculated K_p's assuming the free energy of transfer is directly proportional to the accessible surface area. Constants of proportionality used were 20 and 25 cal mol^{-1} K^{-1}/A^{-2} for the lower and upper lines, respectively.

where $N_{bilayer}$ is the mole fraction peptide associated with the bilayer and $N_{aqueous}$ is the mole fraction peptide in the aqueous phase. The results of experiments aimed at measuring K_p for a homologous series of tripeptides are shown in Table I. The temperature (33 °C) was chosen to ensure that the bilayer is in its fluid state. There is a large change in K_p upon changing the central residue from glycine to tryptophan, while only a very small change is observed upon substitution of alanine for glycine. Figure 1 shows K_p plotted vs. the calculated surface of the central amino acid residue (Chothia, 1976). A monotonic increase of K_p with surface area is observed.

Fluorescence Spectral Changes Induced by Lipid. The spectral properties of the tryptophan indole ring system are known to be strongly dependent upon the local environment of the chromophore (Bell, 1981; Cogwill, 1967). In fluorescence emission spectra of the tryptophan containing tripeptide in the presence of increasing concentrations of DMPC vesicles, the fluorescence intensity increases and the wavelength of maximum intensity (λ_{max}) decreases from 354 nm in the ab-

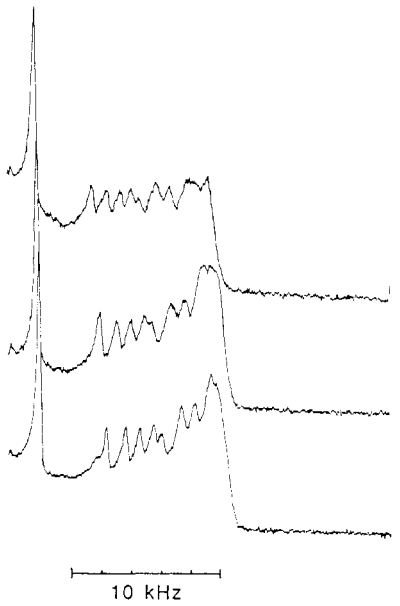

FIGURE 2: ^2H NMR spectra of acyl-chain perdeuterated DMPC liposomes: as the pure lipid (lower trace), with the addition of 20 mol % Ala-Ala-Ala-*O-tert*-butyl (middle trace), and with the addition of 18 mol % Ala-Trp-Ala-*O-tert*-butyl (upper trace). For the sake of clarity, only half of the symmetric powder pattern is shown. Temperature was 30 °C; spectral acquisition parameters are outlined under Experimental Procedures.

Table II: Deuterium NMR Quadrupole Splittings for the Eight Innermost Powder Patterns in Acyl-Chain Perdeuterated DMPC Liposomes with and without the Addition of Peptide[a]

DMPC	A-A-A-*O-tert*-butyl (20 mol %)	A-W-A-*O-tert*-butyl (18 mol %)
3.6	3.3	2.9
12.7	11.8	10.7
15.3	13.9	12.6
17.2	15.9	14.4
19.0	17.7	15.9
20.2	18.7	17.0
22.7	21.1	19.2
av % change	−7.4 ± 0.6	−15.9 ± 1

[a] All quadrupole splittings are expressed in kilohertz. The average percent change is expressed as mean ± SD. The measurements were performed at 30 °C. Figure 2 shows the spectra.

sence of lipid to 345 nm at 5 mM DMPC. A double-reciprocal plot of Ala-Trp-Ala-*O-tert*-butyl fluorescence intensity as a function of DMPC concentration (0.5–5 mM) yields a straight line. Linear least-squares analysis of the data gives slope = 1.76 ± 0.05 mM and ordinate intercept of 0.174 ± 0.04. By use of eq 3, the slope and intercept yield $K_p = 5.5 \times 10^3$ and maximal fluorescence enhancement factor of 6.7. This value of K_p for the tryptophan tripeptide is only marginally larger than that obtained via the hygroscopic desorption method (see Table I and Figure 1). The fact that similar values for the tryptophan tripeptide K_p are obtained with two very different techniques leads us to believe that we can use the K_p data in Table I with some confidence. At the very least the hydroscopic desorption K_p's should accurately reflect the relative propensities of the peptides to partition into the lipid bilayer.

Lipid ^2H NMR Spectral Changes Induced by Peptide. Figure 2 shows ^2H NMR spectra of acyl-chain perdeuterated DMPC liposomes. The methyl and seven methylene powder patterns are easily discernible, while all the other methylenes are crowded together in the outer edge of the spectra. Numerical values for the residual quadrupole splittings ($\Delta\nu_q$) of the eight discernible powder patterns are given in Table II.

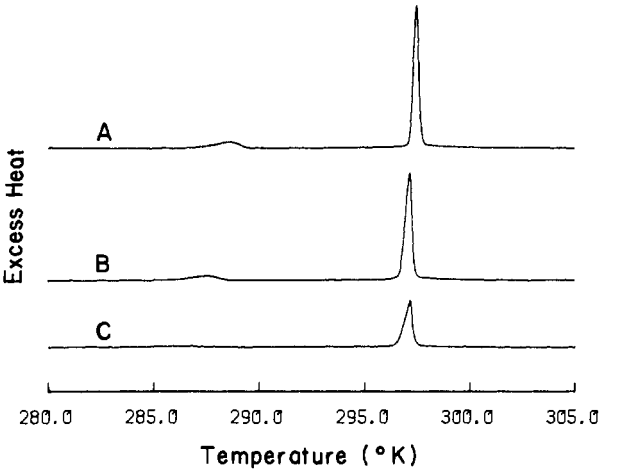

FIGURE 3: DSC thermograms of multilamellar suspensions of DMPC with and without the addition of peptide: A, pure lipid; B, 15 mol % Ala-Gly-Ala-*O-tert*-butyl; and C, 15 mol % Ala-Ala-Ala-*O-tert*-butyl.

Table III: Calorimetric Transition Temperatures and Enthalpies for Peptide/DMPC Liposome Mixtures

sample	mol % peptide	T_m (°C)[a]	ΔH (kcal/mol of DMPC)[b]
DMPC	0	24.5	6.3
A-G-A-*O-tert*-butyl	5	24.1	6.2
	10	24.2	5.9
	15	24.0	5.7
A-A-A-*O-tert*-butyl	5	23.8	5.5
	10	23.8	5.5
	15	23.8	5.1
A-F-A-*O-tert*-butyl	5	23.2	6.8
	10	22.3	6.1
	15	19.6	5.7
A-W-A-*O-tert*-butyl	5	23.1	6.8
	10	21.8	7.1
	15	19.9	6.4

[a] T_m is the temperature of maximal heat absorption. [b] ΔH is enthalpy associated with the higher temperature heat absorption event seen in Figures 3 and 4. Error associated with this measurement is on the order of 10%.

The addition of peptide causes a general decrease in $\Delta\nu_q$ which is essentially the same for the methyl and all of the methylenes. The fractional change in $\Delta\nu_q$ induced by A-A-A-*O-tert*-butyl is 7%, while that induced by A-W-A-*O-tert*-butyl is 16%. In contrast, large amounts of hexane can be incorporated into fluid-phase DMPC bilayers with no appreciable change in the lipid ordering (Jacobs & White, 1984). Due to the large number of variables, no attempt was made to fit computer simulations to these spectra. Nevertheless, visual examination of the spectra in Figure 2 indicates that no gross changes in line width accompanies the introduction of large amounts of either of the tripeptides into the bilayer.

Calorimetry. DSC thermograms of pure DMPC liposomes and liposomes with 15 mol % A-G-A-*O-tert*-butyl or A-A-A-O-*tert*-butyl are shown in Figure 3. The addition of either of these peptides causes a small decrease in T_m along with a decrease in intensity and concomitant broadening of the bilayer gel to liquid-crystalline phase transition. Little or no change in enthalpy is observed (see Table III).

Much more dramatic changes in the thermal properties of the bilayer are induced by the addition of the tripeptides that contain aromatic side chains. Figures 4 shows the effects of increasing amounts of tripeptide on the lipid bilayer phase transition. The effects of the phenylalanyl- and tryptophan-containing peptide are qualitatively similar (data not shown

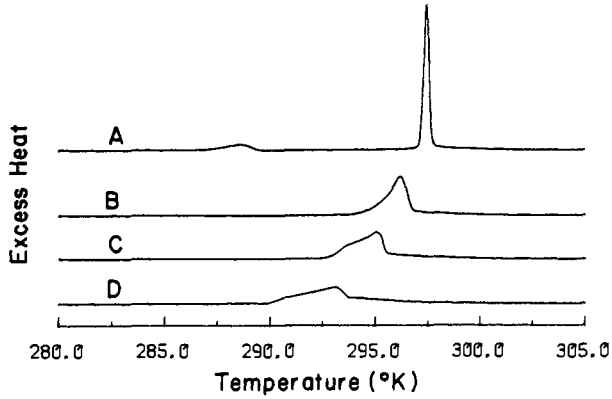

FIGURE 4: DSC thermograms of multilamellar suspensions of DMPC containing various amounts of Ala-Trp-Ala-*O-tert*-butyl: A, no peptide; B, 5 mol %; C, 10 mol %; D, 15 mol %.

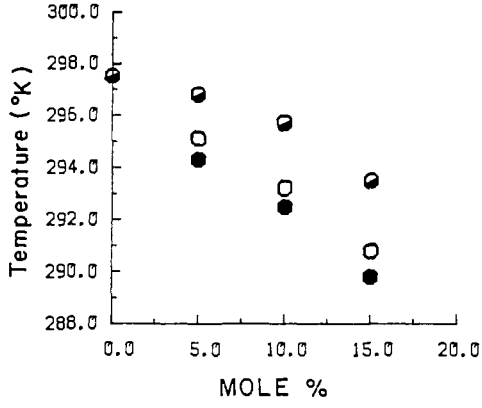

FIGURE 5: Plots of the onset and completion temperatures of the high-temperature heat absorption event in the thermograms shown in Figures 3 and 4. Open circles: Ala-Phe-Ala-*O-tert*-butyl/DMPC mixtures. Closed circles: Ala-Trp-Ala-*O-tert*-butyl/DMPC mixtures. Heat absorption onset and completion temperatures were determined as described by Mabrey and Sturtevant (1976), except that no correction was made for the finite width of the pure DMPC–bilayer phase transition.

for the phenylalanyl peptide). The addition of small amounts of either peptide causes the pretransition to disappear and the main transition to broaden considerably with a shift to lower temperatures. At larger concentrations of peptide the endotherms become quite broad and exhibit a complex multipeak line shape. Taking the base-line intercept of the relatively sharp features at the low- and high-temperature extremes of the endotherms (Mabrey & Sturtevant, 1976) yields the plot shown in Figure 5. The high-temperature completion points are essentially the same for both peptides. The low-temperature onset points occur at lower temperatures for the tryptophan-containing peptide than for the phenylalanyl-containing peptide.

It should be noted that all of the mole percent values quoted above are based on the total amount of peptide in the sample. Since there are physical differences between vesicular lipid bilayers and multilamellar lipid bilayers (Sheetz & Chan, 1972), it is not a priori certain that amphiphiles will partition into both systems to the same extent. Nevertheless, if the partition coefficients measured for vesicular DMPC bilayers are assumed to also apply to the liposome systems used in the NMR and DSC experiments, the calculation of the amount of peptide in the bilayer and in the aqueous phase is straightforward. In the NMR experiments the amounts of peptide in the bilayer become 11 and 14 mol %, respectively, for the alanyl- and tryptophan-containing peptides. In the DSC experiments with 5, 10, and 15 mol % peptide added, for the glycyl peptide 2.8, 6.4, and 9.5 mol % are incorporated, for the alanyl peptide 2.5, 7.5, and 10.2 mol % are incorporated, for the phenylalanyl peptide 4.7, 9.2, and 14.3 mol % are incorporated, while for the tryptophan peptide 5, 10, and 15 mol % are incorporated into the bilayer.

Discussion

We have examined some of the microscopic and thermodynamic properties of lipid bilayers with admixtures of relatively small and simple peptides. All of the tripeptides have the same amino-terminal alanyl and carboxy-terminal alanyl *tert*-butyl ester, differing only in the central amino acid residue. This initial study focuses on four uncharged residues that span the Tanford–Nozaki and other hydrophobicity scales (Tanford, 1962; Nozaki & Tanford, 1971; Charton & Charton, 1982; Eisenberg, 1984) and are of very different sizes: glycyl, alanyl, phenylalanyl, and tryptophan. Following Jahnig (1983) and Engelman and Steitz (1981), we express the total free energy change associated with the incorporation of a peptide into the membrane as

$$\Delta G^\circ = \Delta G_c + \Delta G_h + \Delta G_w + \Delta G_p + \Delta G_l$$

the sum of contributions from peptide conformational change (ΔG_c), alterations in hydrogen bonding (ΔG_h), the hydrophobic effect (ΔG_w), the protein immobilization effect (ΔG_p), and lipid perturbation effects (ΔG_l). In this peptide series the only alteration in chemical structure occurs in the central amino acid side chain. Thus, one would hope to be able to attribute differences in the measured partition coefficients for these peptides in terms of free energy contributions arising from the central amino acid side chains. This is appropriate only if each of the peptides adopts the same conformation (or samples a similar portion of conformational space) in the aqueous phase and, similarly, if the conformation of the peptides is the same in the bilayer. This is tantamount to making the assumption of group additivity (Tanford, 1980), i.e., that the different portions of the molecule make independent contributions to the free energy expression noted above. Making this assumption, we can directly compare the partition coefficients in Table I with various hydrophobicity scales of amino acid residues. As discussed by Eisenberg (1984) and Charton and Charton (1982), the different scales often reflect different properties of the amino acids. We emphasize that these scales are aimed at describing the folding process of globular proteins in which the peptide chain comes to interact with itself rather than the aqueous environment, whereas the process of interest here is the insertion of the peptide chain into a liquid-crystalline hydrophobic environment—the bilayer membrane. According to various hydrophobicity scales the Ala and Gly residues are similar and much less hydrophobic than the Trp and Phe residues. This rough ranking is reflected in the partition coefficients for the tripeptides (see Figure 1). Several of the scales rank tryptophan less hydrophobic than phenylalanine and some rank it less hydrophobic than glycine (Kyte & Doolittle, 1982). In the tripeptide series the Trp-containing peptide is clearly the most hydrophobic. Considering the variation among the hydrophobicity scales, there is reasonable agreement between them and the tripeptide partition data.

Several models describing protein incorporation into membranes make the simplifying assumption that ΔG_w is directly proportional to the interfacial area (Jahnig, 1983; Engelman & Steitz, 1981; von Heijne & Blomberg, 1979). From analyses of amino acid and hydrocarbon partitioning between water and organic solvents, a free energy per area of 20–25 cal/mol^{-1}/A^{-2} is obtained (Richards, 1977; Reynolds et al., 1974). The two lines in Figure 1 are the K_p's calculated by

using these values for ΔG_w. To facilicate comparison, the peptide K_p's have been shifted so that the glycyl peptide value lies at the origin. The measured K_p's are all significantly lower than those calculated. Taken at face value this indicates that the hydrophobic amino acid side chains have significantly less affinity for the bilayer than predicted by this range of ΔG_w. Whether this is due to a breakdown in the group additivity assumption, the fact that the bilayer is a highly anisotropic solvent, or contributions from the other terms in the equation for $\Delta G°$ is not clear at present.

Water to bilayer partition coefficients for a large number of amphiphiles have been measured (e.g., see Table I). The partition coefficients for the glycyl and alanyl peptides are of the same magnitude as those found by Katz and Diamond (1974b) for medium to small size alcohols (see Table I). K_p for the tryptophan-containing tripeptide is close to that found by Surewicz and Epand (1984) for charged pentagastrin analogues, whereas the neutral analogue has a partition coefficient more than an order of magnitude larger. As expected, K_p for fatty acids is much larger than those of the peptides—more than a factor of 2000 larger than the glycyl tripeptide.

The extent of peptide association deduced from the DSC thermograms parallels the partition coefficients measured via the hygroscopic desorption and fluorescence methods. Increasing effects are seen as the central residue is changed from Gly to Ala to Phe to Trp. At the same mole percent peptide the main DMPC phase transition is perturbed only slightly more by the alanyl than the glycyl tripeptide. Neither of these has the dramatic effects induced by the phenylalanyl and tryptophan peptides. The tryptophan peptide induces the formation of a slightly broader transition at a somewhat lower temperature than is observed upon addition of the same amount of the phenylalanyl tripeptide. In thermograms of some mixtures of short peptides (e.g., glucagon and pentagastrin) and lipid there is an endotherm attributable to undisturbed lipid (Epand & Sturtevant, 1984). No undisturbed lipid melting endotherm is observed in these mixtures, indicating homogeneous lipid/peptide mixtures.

The heat absorption peaks in thermograms of two-component lipid bilayers can be interpreted in terms of the phase diagram for the mixture (Mabrey & Sturtevant, 1976). Figure 5 shows such a plot derived from thermograms of DMPC/A-F-A-*O-tert*-butyl and DMPC/A-W-A-*O-tert*-butyl mixtures. The liquidus curve delineates the boundary between the high-temperature single-phase fluid and lower temperature two-phase fluid plus solid region (Findley, 1951). This boundary (locus of high-temperature points in Figure 5) is coincident for the phenylalanyl and tryptophan peptides. The solidus curve (marking the two-phase and single solid-phase boundary) occurs at slightly lower temperatures for the tryptophan than the phenylalanyl peptide. This implies that the larger tryptophan peptide destabilizes the gel-phase lipid more than does the phenylalanyl-containing peptide. Thus, the Trp- and Phe-containing tripeptides induce a phase separation in the plane of the membrane in which a peptide-poor gel phase is in equilibrium with a peptide-rich liquid-crystalline phase. A similar two-phase region is seen in mixtures of DPPC and a leucine oligomer, K_2-G-L_n-K_2-A-amide, where n is either 16 or 24, although in this system the phenomenon is shifted to much smaller peptide concentrations: 1–6 mol % (Davis et al., 1983; Huschilt et al., 1985). At the concentrations examined, the Gly- and Ala-containing peptides act as simple impurities—broadening and lowering the temperature of the phase transition.

It is useful to compare the tripeptide/bilayer mixtures with mixtures of lipids with more structurally similar molecules. Alkanes, fatty acids, and cholesterol are each archetypal hydrophobic molecules that form characteristically different types of mixtures with lipid bilayers. The type of phase behavior, morphology, and structural characteristics of alkane/lipid bilayer mixtures are strongly dependent upon the alkane length and type of lipid (Jacobs & White, 1984; McIntosh & Costello, 1981; McIntosh et al., 1980). For example, in the hexane/DMPC mixture there is a solid–solid-phase separation of hexane-rich and hexane-poor solid phases at temperatures below the pure lipid T_m (Jacobs & White, 1984), while the phase transition temperature of hexadecane/DPPC bilayer mixtures increased with increasing alkane concentration (McIntosh et al., 1980). Fatty acids also tend to raise the lipid bilayer phase transition temperature (Mabrey & Sturtevant, 1977). Mixtures of fatty acids in lipid bilayers appear to form sharp melting 1:2::lipid:fatty acid complexes where nonideal mixing is observed between the complex and free lipid when the fatty acid is the same length or longer than the lipid acyl chain (DPPC–palmitic acid and DPPC–stearic acid mixtures) while shorter fatty acids form nearly ideal bilayer mixtures (Schullery et al., 1981). Mixtures of cholesterol and phospholipids exhibit quite complex multipeak DSC thermograms which are difficult to interpret in terms of a phase diagram (Estep et al., 1978; Mabrey et al., 1978). Magnetic resonance experiments indicate that cholesterol has a general plasticizing effect on the lipid bilayer (Brown & Seelig, 1978; Oldfield et al., 1978). In terms of their phase diagrams, the tripeptide/bilayer mixtures we have examined exhibit relatively simple behavior: all appear to mix ideally in the bilayer, no stoichiometric complexes are observed, the less hydrophobic peptides act as simple impurities, and the more hydrophobic ones induce phase separations. Whether this is a general feature of bulky amphipathic molecules associated with the bilayer or a specific characteristic of these peptides in the DMPC bilayer we do not know. To address this question, we are examining mixtures of this series of peptides and longer model peptides (e.g. Ala_4-X-Ala_4-*O-tert*-butyl) in a variety of types of lipid bilayers (e.g., lipids with unsaturated acyl chains and different types of head groups).

Although this investigation does not directly address the question of where the bilayer-associated tripeptides are located (e.g., between the monolayers, aligned with the acyl chains, and/or adsorbed at the lipid/water interface), the fluorescence and ^2H NMR spectroscopic data are suggestive. The blue shift in λ_{max} and increase in intensity of the tryptophan fluorescence upon lipid binding indicate that the fluorophore is being transferred to a solvent of low polarity (Cowgill, 1967; Bell, 1981), i.e., somewhere in the interior of the lipid bilayer. The ^2H NMR lipid spectra undergo an overall contraction upon peptide binding, implying a general disordering of the bilayer induced by both the alanyl- and tryptophan-containing peptides [see Davis (1983), Jacobs and Oldfield (1981), or Seelig (1977) for details concerning the interpretation of lipid bilayer ^2H NMR spectra]. This overall disordering is, again, consistent with the peptides being located in the bilayer interior. These effects and the phase separation seen in the DSC thermograms are most easily explained by assuming that the peptides reside within the bilayer hydrocarbon core with the charged amino terminus at the bilayer/water interface.

CONCLUSION

Relatively simple peptides of the form Ala-X-Ala-*O-tert*-butyl partition into the lipid bilayer. The fashion and extent of the interaction are strongly dependent upon the identity of

the central amino acid residue. Fluorescence, ^2H NMR, and DSC experiments indicate that the peptides are probably located in the bilayer interior. Thus, peptides of this form can serve as easily modifiable model systems with which to study the details of (1) how the bilayer environment affects amino acid side chain motion, (2) how the bilayer organization is affected by different side chains, and (3) how amino acid substitution affects peptide partitioning into the bilayer membrane. If one is interested in modeling the insertion of peptides into membranes (Engelman & Steitz, 1981; von Heijne & Blomberg, 1979; Sabitini et al., 1982; Wickner, 1979), quantitative information concerning the transfer of amino acid residues on peptides into the bilayer membrane is needed. Although assumptions concerning conformational changes need clarification, the use of a simple homologous series of peptides allows us to examine bilayer–amino acid interactions where the amino acid is in a "natural" environment (i.e., attached via peptide bonds to two adjacent amino acids). Detailed NMR and neutron diffraction experiments with specifically labeled peptides are under way which should unequivocally answer the question of the location(s) of these peptides in the bilayer and probe questions of peptide conformation in the bilayer vs. aqueous solvent systems.

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Registry No. DMPC, 18194-24-6; A-G-A-*O-tert*-butyl, 100859-29-8; A-A-A-*O-tert*-butyl, 65356-57-2; A-F-A-*O-tert*-butyl, 100859-30-1; A-W-A-*O-tert*-butyl, 100859-31-2.

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Characterization of Glucagon Receptors in Golgi Fractions of Rat Liver: Evidence for Receptors That Are Uncoupled from Adenylyl Cyclase

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ABSTRACT: Glucagon receptors have been identified and characterized in intermediate (G_i) and heavy (G_h) Golgi fractions from rat liver. At saturation, plasma membranes bound 3500 fmol of hormone/mg of membrane protein, while G_i and G_h bound 24 and 60 fmol of ^{125}I-glucagon/mg of protein, respectively. Half-maximal saturation of binding to plasma membranes, G_i, and G_h occurred at approximately 4, 10, and 20 nM ^{125}I-glucagon, respectively. Trichloroacetic acid precipitation of intact, but not degraded, glucagon was used to correct binding isotherms for hormone degradation. After such correction, half-maximal saturation of binding to plasma membranes, G_i, and G_h was observed in the presence of approximately 2, 7, and 14 nM hormone, respectively. After 90 min of dissociation in the absence of guanosine 5′-triphosphate (GTP), 86% of ^{125}I-glucagon remained bound to plasma membranes, whereas only 42% remained bound to Golgi membranes. GTP significantly increased the fraction of ^{125}I-glucagon released from plasma membranes but only slightly augmented the dissociation of hormone from Golgi fractions. ^{125}I-Glucagon/receptor complexes solubilized from plasma membranes fractionated by gel filtration as high molecular weight (K_{av} = 0.16), GTP-sensitive complexes and lower molecular weight (K_{av} = 0.46), GTP-insensitive complexes. ^{125}I-Glucagon complexes solubilized from Golgi membranes fractionated almost exclusively as the lower molecular weight species. The lower affinity of Golgi than plasma membrane receptors for hormone, the ability of glucagon to stimulate plasma membrane, but not Golgi membrane, adenylyl cyclase, and the near absence of high molecular weight, GTP-sensitive complexes in solubilized Golgi membranes demonstrate that plasma membrane contamination of Golgi fractions cannot account for the ^{125}I-glucagon binding. These observations are novel and significant in demonstrating that (1) Golgi fractions contain specific binding sites for glucagon, (2) such sites bind hormone with lower affinity than plasma membrane receptors, and (3) these sites are uncoupled from other components of adenylyl cyclase.

Receptor/adenylyl cyclase systems are composed of at least three distinct membrane components: a specific receptor such as that for glucagon, guanine nucleotide regulatory proteins (N proteins), and the catalytic subunit of the enzyme adenylyl cyclase (Rodbell, 1980). In such systems, hormone action is initiated by binding to a specific receptor in the plasma membrane. Interactions between occupied receptors and N proteins ("coupling") are obligatory for transmembrane signaling. Guanosine 5′-triphosphate (GTP)[1] binding to the N protein promotes dissociation of the occupied receptor from the N protein and thereby diminishes the affinity of receptor for hormone. The activated N protein that results from this process then stimulates cAMP production by the enzyme until the bound GTP is hydrolyzed to GDP (Rodbell, 1980; Gilman, 1984).

Hormone and neurotransmitter receptors have been characterized in cell fractions other than the plasma membrane. For example, receptors for insulin and prolactin have been identified in Golgi fractions from rat liver (Bergeron et al., 1973b, 1978; Posner et al., 1979). β-Adrenergic receptors that were uncoupled from other components of the adenylyl cyclase system have been isolated in "light" membranes that could not

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; cAMP, adenosine cyclic 3′,5′-phosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GDP, guanosine 5′-diphosphate; Gpp(NH)p, guanylyl imidodiphosphate; GTP, guanosine 5′-triphosphate; G_i, intermediate Golgi fraction; G_h, heavy Golgi fraction; HBSS, Hank's balanced salt solution; K_D, dissociation constant, Cl_3CCOOH, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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