Peptide Binding to Lipid Bilayers. Nonclassical Hydrophobic Effect and Membrane-Induced p*K* Shifts†

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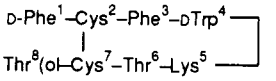
ABSTRACT: The binding of the cyclic peptide (+)-D-Phe[1]–Cys[2]–Phe[3]–D-Trp[4]–(+)-Lys[5]–Thr[6]–Cys[7]–Thr(ol)[8], a somatostatin analogue (SMS 201-995), and the potential-sensitive dye 2-(*p*-toluidinyl)naphthalene-6-sulfonate (TNS) to lipid membranes was investigated with high-sensitivity titration calorimetry. The binding enthalpy of the peptide was found to vary dramatically with the vesicle size. For highly curved vesicles with a diameter of $d \simeq 30$ nm, the binding reaction was enthalpy-driven with $\Delta H \simeq -7.0 \pm 0.3$ kcal/mol; for large vesicles with more tightly packed lipids, the binding reaction became endothermic with $\Delta H \simeq +1.0 \pm 0.3$ kcal/mol and was entropy-driven. In contrast, the free energy of binding was almost independent of the vesicle size. The thermodynamic analysis suggests that the observed enthalpy–entropy compensation of about 8 kcal/mol can be related to a change in the internal tension of the bilayer and is brought about by an entropy increase of the lipid matrix. The "entropy potential" of the membrane may have its molecular origin in the excitation of the hydrocarbon chains to a more disordered configuration and may play a more important role in membrane partition equilibria than the classical hydrophobic effect. The binding of the peptide to the membrane surface induced a p*K* shift of the peptide terminal amino group. Neutral membranes were found to destabilize the NH_3^+ group, leading to a decrease in p*K*; negatively charged membranes, generated an apparent increase in p*K* due to the increase in proton concentration near the membrane surface. No p*K* shifts were seen for TNS. Titration calorimetry combined with the Gouy–Chapman theory can be used to determine both the reaction enthalpy and the binding constant of the membrane-binding equilibrium.

The binding of apolar molecules to lipid membranes is usually considered to be the consequence of the "hydrophobic effect" with the solute inducing a specific ordering of the water molecules in its vicinity. Upon binding to the membrane, water molecules are released from the solute hydration shell, leading to an entropy-driven association reaction. A typical example is the partitioning of *n*-hexane into phospholipid bilayers where the entropy term makes the main contribution to the free energy change of $\Delta F \simeq -6.0$ kcal/mol (Simon et al., 1977, 1979). However, a number of experimental results are contradictory to this entropic interpretation. For example, the transfer of NBD-labeled phosphatidylcholine molecules from water to lipid vesicles exhibits an enthalpy of transfer of −8 kcal/mol, accounting for 80% of the total free energy of transfer (Nichols, 1985). The enthalpic component assumes an even more important role in the membrane-binding reaction of hydrophobic ions. Using high-sensitivity titration calorimetry, we have recently measured exothermic heats of reaction of up to −10 kcal/mol for hydrophobic cations and anions of quite different chemical structure (Seelig & Ganz, 1991). For these molecules the membrane-binding reaction was enthalpy-driven whereas the reaction entropy was zero or even negative. A "nonclassical" hydrophobic effect (Jencks, 1969; Huang & Charlton, 1972) is not unique to membrane systems. Other striking examples are tight apolar complexation processes such as the cyclodextrin or cyclophane inclusion compounds which are also characterized by large exothermic heats of formation and negative reaction entropies, again in sharp contrast to the classical hydrophobic effect (Clark et al., 1988; Smithrud et al., 1991).

Titration calorimetry has also been employed in a number of peptide binding studies, and large exothermic as well as large endothermic heats of reaction have been observed (Epand & Sturtevant, 1981; Massey et al., 1981; Myers et al., 1987; Epand et al., 1990). However, most of these studies were performed with disaturated lipids, and the peptide binding was accompanied by a lipid phase transition or by a change in peptide conformation, masking to a large extent the binding step proper.

Here we have investigated a peptide binding reaction with unsaturated lipids such as 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)[1] and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) in order to eliminate the possibility of a gel-to-liquid crystal phase transition. As a model peptide we have employed the drug SMS 201-995,



which is a somatostatin analog with a biological activity larger than that of somatostatin itself (Maurer et al., 1982). Monolayer studies and fluorescence spectroscopy demonstrated an intercalation of the cyclic peptide between the lipids, but CD spectroscopy revealed only a minor conformational change of the peptide structure (Beschiaschvili & Seelig, 1991). The peptide carries an effective charge of $z \simeq +1.3$, and the apparent binding constant is strongly influenced by the electric surface charge of the membrane. After correcting for electrostatic effects using the Gouy–Chapman theory, the peptide binding can be described with high accuracy by a

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[1] Abbreviations: TNS, 2-(*p*-toluidinylnaphthalene)-6-sulfonate; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; SUV, small unilamellar vesicles.

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Table I: Binding Enthalpies for TNS Binding to POPC Vesicles: Influence of Buffer and Vesicle Size (28 °C)

C_{TNS}[a] (μM)	Influence of Buffer (Sonified POPC Vesicles) buffer	Δh (μcal)	ΔH (kcal/mol)
331	10 mM MOPS, 100 mM NaCl, pH 7.4	−31.4 ± 0.7	−9.5 ± 0.3
432	10 mM phosphate, 100 mM NaCl, pH 7.4	−41.0 ± 0.5	−9.6 ± 0.3
353	10 mM Tris, 100 mM NaCl, pH 7.4	−32.0 ± 0.4	−9.1 ± 0.3

C_{TNS} (μM)	Influence of Vesicle Size[b] size (nm)	pH	Δh (μcal)	ΔH (kcal/mol)
353	30, sonified	7.4	−32.0 ± 0.4	−9.1 ± 0.3
353	50	7.4	−26.4 ± 0.4	−7.5 ± 0.3
353	100	7.4	−26.9	−7.6 ± 0.3
590	100	8.0	−43.5 ± 1.1	−7.4 ± 0.3
590	30, sonified	8.0	−54.8	−9.3
366[c]	100	8.0	−28.1	−7.7 ± 0.3

[a] Concentration of TNS in the injection syringe. [b] All measurements were made with 10 mM Tris/0.1 M NaCl buffer. [c] 10 mM Tris, 0.1 M KCl buffer.

simple surface partition equilibrium with an intrinsic binding constant of $K_p \simeq 36\ M^{-1}$ (Beschiaschvili & Seelig, 1990a). In the present study, the thermodynamics of this peptide–membrane equilibrium were further characterized by measuring the reaction enthalpy and entropy with high-sensitivity titration calorimetry. Unilamellar phospholipid vesicles were employed throughout, and the role of the internal membrane tension was investigated by varying the vesicle diameter from 30 to 400 nm. The data suggest that a peptide-induced area expansion of the lipid phase with a concomitant increase in the lipid chain disorder is a major driving force for the peptide binding to planar bilayers. In addition, the calorimetric results provided evidence for a pK shift of the peptide at the membrane surface which could be quantified by employing buffers of different dissociation enthalpies.

MATERIALS AND METHODS

Chemicals. SMS 201-995 was kindly provided by Sandoz LTD (Basel, Switzerland) and had a purity of better than 95%. The peptide concentration was determined by UV spectroscopy using an absorption coefficient of 5700 M^{-1} cm^{-1} at 280 nm (Holladay et al., 1977). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) were purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification.

Preparation of Lipid Samples. Small unilamellar vesicles of diameter $d \simeq 30$ nm were prepared as follows. The lipids were dissolved in chloroform or dichlormethane, mixed in the desired ratio, and dried over night under high vacuum. Buffer (10 mM Tris, MOPS, or phosphate; 100 mM NaCl) was added to the dry lipid film, and the suspension was vortexed extensively. Next, the lipid dispersions were sonified under a nitrogen atmosphere for 35–45 min (at 10 °C) until an almost clear solution was obtained. Metal debris from the titanium tip was removed by centrifugation in an Eppendorf centrifuge for 10 min.

Unilamellar vesicles of diameter $d \simeq 50$, 100, or 400 nm were obtained by extrusion of multilamellar lipid suspensions through a polycarbonate filter (Mayer et al., 1986). The dried lipid was suspended in 10 mM buffer plus 100 mM NaCl. The lipid suspensions were freeze-thawed for at least three cycles and were vortexed extensively before extrusion. An extruder with a 10-mL barrel from Lipix Biomembranes (Vancouver, BC) was used. Unilamellar vesicles of the desired diameter were extruded under nitrogen pressure (maximum pressure 15 atm) through two stacked polycarbonate filters (Nucleopore) by stepwise decreasing the pore size (d = 400 nm, d = 100 nm, d = 50 nm; three times for each filter), i.e., unilamellar vesicles of d = 50 nm were extruded three times through filters with a pore size of d = 400 nm, three times through filter with d = 100 nm, and finally three times through filter with the pore size d = 50 nm.

High-Sensitivity Titration Calorimeter. Heats of reaction were measured with a Microcal MC-2 high-sensitivity titration calorimeter (Microcal, Northampton, MA) as described previously (Wiseman et al., 1989). Solutions were degassed under vacuum prior to use. The calorimeter was calibrated electrically. The data were acquired by computer using software developed by MicroCal.

RESULTS

TNS Binding to POPC Vesicles. The fluorescent probe TNS adsorbs hydrophobically to phospholipid membranes. The thermodynamic binding parameters have been derived from gel filtration studies (Huang & Charlton, 1972) and from measurements of the electrophoretic mobility (McLaughlin & Harary, 1976). After correcting for electrostatic effects, the intrinsic binding/association constant of TNS to phosphatidylcholine vesicles was determined as $5 \times 10^3\ M^{-1}$ at 25 °C. The temperature dependence of the binding constant was also measured, yielding a reaction enthalpy of $\Delta H = -9.5$ kcal/mol (Huang & Charlton, 1972). Using high-sensitivity titration calorimetry, we have directly measured the binding enthalpy of TNS to sonified POPC vesicles as $\Delta H = -9.4$ kcal/mol, confirming the earlier gel filtration results (Seelig & Ganz, 1991). In the present study, TNS is used as a standard in order to critically evaluate the peptide binding data.

TNS is negatively charged and the sulfate group has a pK value of pK < 2. At physiological pH, binding of TNS to phospholipid membranes is expected not to be accompanied by a change in the protonation state. This was verified experimentally by measuring the binding/association enthalpy of TNS to POPC unilamellar vesicles in different buffers. Since chemically different buffers have different dissociation enthalpies, protonation or deprotonation reactions of TNS should entail quite different enthalpy changes. The experimental data for phosphate (ΔH_{diss} = 1.22 kcal/mol), MOPS (ΔH_{diss} = 5.29 kcal/mol), and Tris buffer (ΔH_{diss} = 11.51 kcal/mol) are summarized in Table I [buffer dissociation enthalpies, ΔH_{diss}, are taken from Morin and Frei (1991)]. Inspection of Table I demonstrates that the binding enthalpy

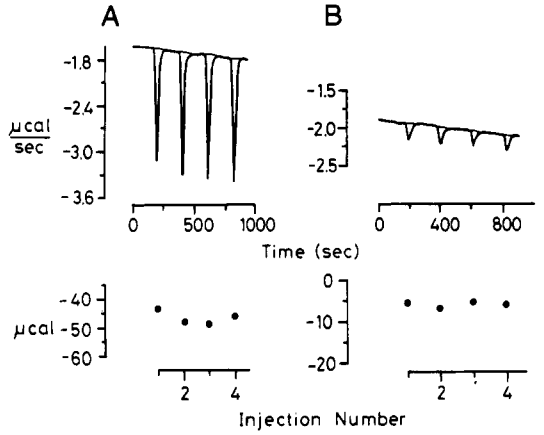

FIGURE 1: Titration calorimetry of sonified unilamellar vesicles (d = 30 nm) composed of POPG/POPC (25/75 mol/mol) (panel A) and of extruded unilamellar vesicles (d = 100 nm) of same chemical composition (panel B) with a SMS 201-995 solution. Three (A) or four microliters (B) of a 1.6 mM solution of SMS 201-995 in buffer was injected into the calorimeter cell (1.27 mL) containing a 26.4 mM lipid dispersion in the same buffer (27 °C; 10 mM phosphate, 100 mM NaCl, pH 6.25). The upper part of the figure shows the calorimeter tracings; the bottom part yields the heat of reaction as evaluated from the areas under the calorimeter tracings. The binding enthalpy becomes less exothermic with increasing vesicle size.

of TNS is $\Delta H \simeq -9.4 \pm 0.3$ kcal/mol for all three buffers investigated.

As a second parameter, we have measured the influence of vesicle size and membrane curvature on the TNS binding enthalpy. These data are also included in Table I. Small unilamellar vesicles (SUV) of diameter $d \simeq 30$ nm were produced by sonification; larger vesicles were made by the extrusion method (diameters 50 and 100 nm). For the 30-nm vesicles, the reaction enthalpy was found to be $\Delta H \simeq -9.4$ kcal/mol. For the two larger vesicle sizes, the magnitude of ΔH decreased, and the enthalpy was $\Delta H \simeq -7.6$ kcal/mol. The free energy of binding was $\Delta G \simeq -7.4$ kcal/mol as calculated from the binding constant (McLaughlin & Harary, 1976).

A similar dependence of the binding enthalpy on the vesicle size has been observed previously for other hydrophobic drugs such as amlodipine (Bäuerle & Seelig, 1991), tetraphenylborate (Seelig & Ganz, 1991), chlorpromazine, and fluxpentixol (Frenzl, 1992). In all cases studied, the ΔH values were distinctly negative for sonified SUVs and became somewhat less negative (by about 2 kcal) for 100- or 400-nm vesicles. However, the change in ΔH with vesicle size was always less than 30%.

Peptide Binding at pH 6.25. Titration of SMS 201-995 with NaOH in 100 mM NaCl revealed a pK value of 7.2 for the N-terminal (D-Phe[1]) amino group. In contrast, the pK of the Lys[5] amino group is pK > 9. Since the C-terminal group is Thr[8](ol), the peptide carries an average charge of $\langle z \rangle \approx +1.9$ at pH 6.25, which is close to its maximum charge $\langle z \rangle = 2$. At pH 7.4, the peptide charge is reduced to $\langle z \rangle \simeq +1.4$.

Titration calorimetry was performed at pH 6.25 and pH 7.4 using three different buffers, namely phosphate (pK = 7.2), MOPS (pK = 7.2), and TRIS (pK = 8.3) buffer. As is obvious from the pK values, only the phosphate and MOPS buffers have sufficient buffer capacity at both pH values whereas TRIS cannot be used at pH 6.25.

Figure 1 shows the result of a titration study at pH 6.25 for two different vesicle sizes. The calorimeter cell contained sonified or extruded lipid vesicles composed of POPC/POPG (75/25 mol/mol). Each calorimeter trace corresponds to the injection of 4 μL (3 μL) of a 1.6 mM peptide solution into an extruded (sonified) vesicle suspension. Due to the large negative surface charge on the membrane and the $z = +1.9$ charge of the peptide, virtually all injected peptide is bound to the membrane surface, and the same heat of binding is released in each reaction step. The binding enthalpy can be calculated as the ratio of the measured heat change and the amount of added peptide. As a control, the same peptide solution was injected into buffer without lipids. The heat of dilution was found to be negligible.

Table II: Binding Enthalpies of SMS 201-995 Binding to Mixed POPC/POPG (75/25 mol/mol) Membranes as a Function of Buffer Composition and Vesicle Size at pH 6.25

vesicle size (nm)	ΔH (MOPS)[a] (kcal/mol)	ΔH (phosphate)[a] (kcal/mol)
30[b]	−7.2 ± 0.3	−7.4 ± 0.3
100[c]	−1.5 ± 0.3	−1.4 ± 0.3

[a] Buffer composition: 10 mM phosphate or MOPS, 100 mM NaCl, pH 6.3. Lipid composition: POPC/POPG (75/25 mol/mol). [b] Produced by sonification. [c] Produced by extrusion through a 100-nm polycarbonate filter.

Figure 1 reveals a strong dependence of the binding enthalpy, ΔH, on the vesicle size. For sonified SUVs with a vesicle diameter of ≃30 nm, the binding enthalpy is about −7.3 kcal/mol. In contrast, ΔH is distinctly less negative for extruded vesicles with 100-nm diameter and amounts to −1.4 kcal/mol. At the same time, the free energy, ΔG, changes by less than 1 kcal/mol as the vesicle size increases (cf. below). The binding enthalpy is independent of the buffer composition. Changing the buffer from phosphate to MOPS led to practically identical calorimeter tracings and identical ΔH values. The experimental data are summarized in Table II.

In the above experiments, a small amount of peptide was added to a large excess of lipid vesicle suspension, and all peptide was immediately and completely bound to the lipid surface. This allowed the direct determination of ΔH. However, if the experiment is performed in the reversed order, i.e., by titration of lipid vesicles into a peptide solution, it is also possible to determine the binding constant K. The outcome of such an experiment is illustrated in Figure 2A. The calorimeter cell (V = 1.278 mL) contained a 50 μM SMS 201-995 solution in buffer (10 mM phosphate, 100 mM NaCl, pH 6.25). Each calorimeter trace corresponds to the injection of 14 μL of sonified lipid vesicles [POPC/POPG (75/25 mol/mol)]. The lipid concentration was 55 mM; however, only 60% of the lipid (the outer surface of the lipid vesicle) was available for peptide binding. Figure 2A demonstrates that the heat of reaction decreases with consecutive injections since less and less peptide is available for binding. Figure 2B then shows the cumulative reaction enthalpy as a function of the number of injections. The solid line in Figure 2B corresponds to the best theoretical fit to the data calculated with ΔH = −7.3 kcal/mol, an intrinsic binding constant of $K = 90\ M^{-1}$, and an effective peptide charge $\langle z \rangle = 1.9$. The ΔH evaluated from this titration curve is in excellent agreement with the results shown in Figure 1 and summarized in Table II. The model used for this calculation will be described below.

Peptide Binding at pH 7.4. Influence of Vesicle Size. As mentioned above, the pK of the N-terminal D-Phe amino group of SMS 201-995 is 7.2. At pH 7.4 this amino group is only 40% protonated, and the total effective charge of the peptide is $\langle z \rangle \simeq 1.4$. Measuring the binding enthalpy at pH 7.4 reveals again a distinct dependence on the size of the lipid vesicles as demonstrated in Figure 3 for three different membrane compositions. A large negative reaction enthalpy

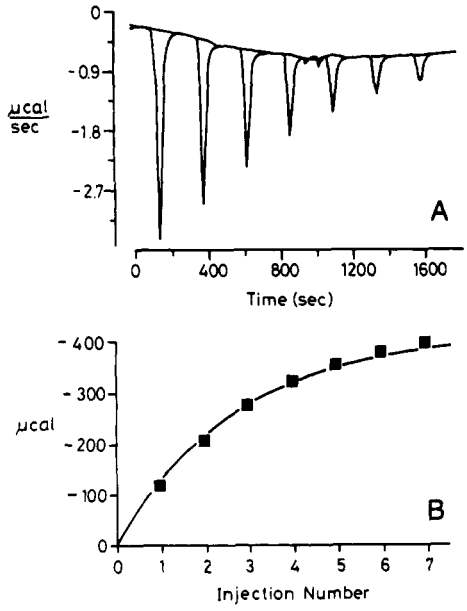

FIGURE 2: Titration calorimetry of a SMS 201-995 solution (50 μM) with 55 mM sonified small unilamellar POPC/POPG (75/25 mol/mol) vesicles in buffer (27 °C; 10 mM phosphate, 100 mM NaCl, pH 6.25). Fourteen microliters of the lipid dispersion was injected into the calorimeter cell containing SMS 201-995 in buffer. The upper part of the figure shows the calorimeter tracings; the bottom part yields the cumulative reaction enthalpy as a function of the number of injections. The solid line corresponds to the best theoretical fit to the data calculated with $\Delta H = -7.3$ kcal/mol, an intrinsic binding constant $K_P = 90$ M^{-1}, and an effective charge $\langle z \rangle = 1.9$.

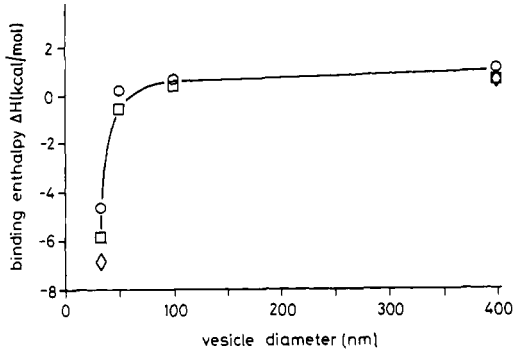

FIGURE 3: Peptide binding enthalpy as a function of vesicle size. Membrane composition: (◇) 100% POPC; (○) 100% POPG; (□) POPC/POPG (75/25 mol/mol) (27 °C, 10 mM Tris, 100 mM NaCl, pH 7.4).

was found for sonified vesicles with ΔH ranging from –4.6 kcal/mol for 100% POPG to –6.8 kcal/mol for 100% POPC. However, a small increase in vesicle size to 50 nm caused a steep decrease in the magnitude of ΔH, and the reaction enthalpy became $\Delta H \sim 0$ kcal/mol. A further increase in vesicle size to 100 and 400 nm even led to positive ΔH values, indicating a small endothermic reaction for the less curved membranes.

The ΔH values summarized in Figure 3 were obtained by different methods of evaluation. For 100% POPG membranes (which are highly charged), it was again safe to assume that all peptide was bound to the lipid surface, and the calculation of the binding enthalpy was straightforward. ΔH increased from –4.6 kcal/mol for sonified POPG vesicles to 0.5 kcal/mol for 50-nm vesicles and to +1.1 kcal/mol for 400-nm vesicles.

For mixed POPC/POPG (75/25 mol/mol) vesicles, the binding situation was slightly different. Due to the reduced

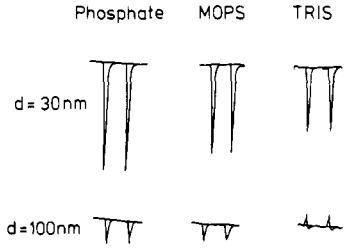

FIGURE 4: Peptide binding enthalpy as a function of the vesicle size and the buffer composition at pH 7.4. Membrane composition: POPC/POPG (75/25 mol/mol) at a concentration of ~25 mM. Each injection corresponds to an increase in the peptide concentration in the calorimeter cell by 5 μM (10 μM for vesicles with $d = 100$ nm in Tris).

charge of the peptide at pH 7.4, most but not all of the peptide was bound to the membrane surface. The exact ΔH values had thus to be determined via titration experiments analogous to that shown in Figure 2. In three separate titrations with varying lipid and protein concentrations, identical parameters were derived for *sonified* vesicles, namely, an intrinsic binding constant $K = 100 \pm 10$ M^{-1}, an average charge $\langle z \rangle = 1.3$, and a binding enthalpy $\Delta H = -6.3$ kcal/mol. The latter value was confirmed by titrating peptide into an excess of lipid vesicles under conditions where 95% of the peptide was bound. The directly measured ΔH was -5.9 ± 0.1 kcal/mol, which corrected for incomplete binding yielded $\Delta H = -6.2$ kcal/mol.

For *large* POPC/POPG (75/25) vesicles with a diameter of $d = 400$ nm, the binding enthalpy was found to be close to zero. Here the amount of bound peptide was determined via a physical separation of the membrane phase and the solution phase. After several injections of peptide into the vesicle suspension and measurement of the heat consumed, the suspension was removed from the calorimeter cell, centrifuged at 300000*g* for 2 h, and the concentration of free peptide in the supernatant determined by UV spectroscopy. The difference between the initial concentration and the free concentration provided the amount of bound peptide. The intrinsic binding constant was calculated as $K = 33$ M^{-1} and the corrected binding enthalpy as $\Delta H \approx +0.8$ kcal/mol, using $\langle z \rangle = 1.3$.

For *sonified* vesicles consisting of 100% POPC, the binding parameters were evaluated in a titration experiment leading to $K = 170$ M^{-1}, $\langle z \rangle = 1.3$, and $\Delta H = -6.8$ kcal/mol, whereas for *large* POPC vesicles (400 nm) the amount of bound peptide was determined by the centrifugation assay described above. ΔH was found to be +0.7 kcal/mol with $K = 110$ M^{-1} and $\langle z \rangle = 1.3$.

Peptide Binding at pH 7.4. Influence of Buffer and Lipid Composition. The second unusual phenomenon observed at pH 7.4 was the distinct influence of the buffer composition on the measured binding enthalpy. Figure 4 summarizes titration experiments with vesicle suspensions composed of POPC/POPG (75/25 mol/mol), prepared either by sonification ($d \simeq 30$ nm) or by extrusion ($d \simeq 100$ nm). For both types of vesicles, the figure demonstrates a remarkable change in the measured binding enthalpy. For vesicles with $d \simeq 100$ nm, the binding enthalpy changes, in fact, from exothermic in the phosphate and MOPS buffers to endothermic in Tris buffer. Figure 5 (lower panel) then shows the variation of the measured binding enthalpies with the buffer dissociation enthalpies. For both sizes of POPC/POPG vesicles, a linear relationship between the peptide binding enthalpy, ΔH, and the buffer dissociation enthalpy, ΔH_{diss}, is observed with

$$\Delta H = -13.4 + 0.626\Delta H_{diss} \text{ (kcal/mol)} \quad (1)$$

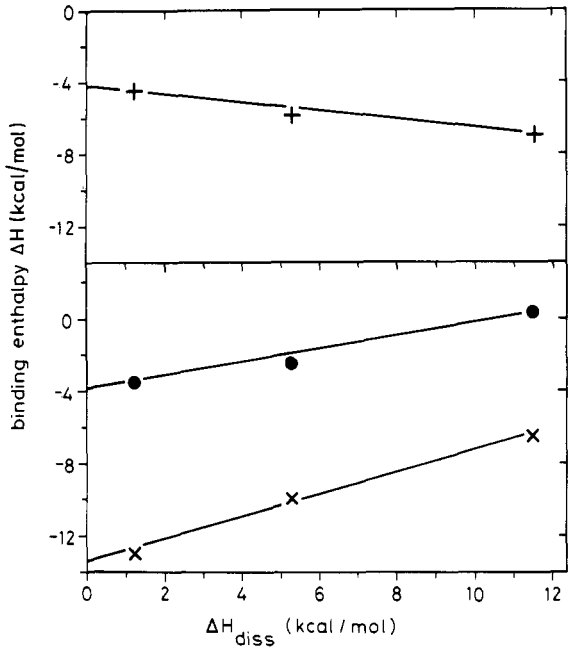

FIGURE 5: Variation of the peptide binding enthalpies with the buffer dissociation enthalpies: (+) pure POPC vesicles, sonified, $d \simeq 30$ nm; (×) POPC/POPG vesicles (75/25 mol/mol), sonified, $d \simeq 30$ nm; (●) POPC/POPG vesicles (75/25 mol/mol), extruded, $d = 100$ nm. Measurements were made in Tris, $\Delta H_{diss} = 11.51$ kcal/mol; MOPS, $\Delta H_{diss} = 5.29$ kcal/mol; phosphate, $\Delta H_{diss} = 1.22$ kcal/mol (Morin & Freire, 1991). All buffers were at pH 7.4 plus 100 mM NaCl (27 °C).

for sonified vesicles and

$$\Delta H = -4.1 + 0.38\Delta H_{diss} \text{ (kcal/mol)} \quad (2)$$

for 100-nm vesicles. The positive slope indicates an *uptake* of protons by the peptide upon membrane binding.

If the same titration experiments are repeated with neutral POPC vesicles, a different result is observed (Figure 5, upper panel). The slope of the ΔH vs ΔH_{diss} plot becomes negative according to

$$\Delta H = -4.2 - 0.237\Delta H_{diss} \text{ (kcal/mol)} \quad (3)$$

(sonified vesicles) indicating a *release* of protons.

Taken together, these results provide experimental evidence for two different types of pK shifts of the Phe[1] terminal amino group which will be discussed in more detail below.

It should be noted that the quantitative results as summarized in eqs 1–3 critically depend on the proper evaluation of dissociation enthalpies as quoted in Morin and Frei (1991). Any deviations in ΔH_{diss} will automatically change the slopes in Figure 5.

Figure 6 finally demonstrates the influence of lipid composition on the measured ΔH (in Tris buffer) of the peptide–lipid binding (sonified vesicles). ΔH increases almost linearly with the POPG content from $\Delta H = -6.8$ kcal/mol for pure POPC to –4.7 kcal/mol for pure POPG membranes.

DISCUSSION

Binding Model. A membrane surface with an electric charge density σ gives rise to a surface potential ψ_0. The quantitative relation between the two parameters is provided by the Gouy–Chapman equation [for reviews, see Aveyard and Haydon (1973) and McLaughlin (1977, 1989)]:

$$\sigma^2 = 2000\epsilon_0\epsilon_r RT\sum C_{i,eq}(e^{-z_i F_0\psi_0/RT} - 1) \quad (4)$$

where $C_{i,eq}$ is the concentration of the ith electrolyte in the bulk aqueous phase (in moles per liter), z_i the signed valency

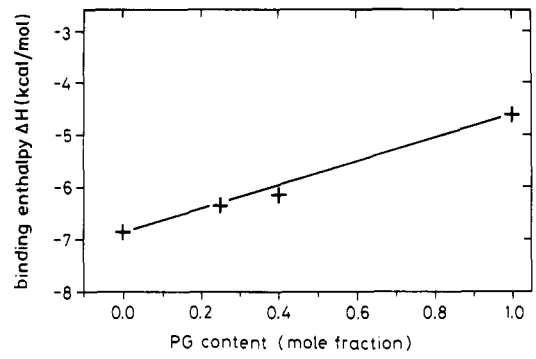

FIGURE 6: Variation of the peptide binding enthalpies of SMS 201-995 with the lipid composition using sonified unilamellar vesicles (d = 30 nm, 27 °C; 10 mM Tris, pH 7.4, 100 mM NaCl).

of the ith species, ϵ_0 the electric permittivity of free space, ϵ_r the dielectric constant of water, F_0 the Faraday constant, and T the absolute temperature.

The effect of the surface potential is to change the ion concentration at the lipid–water interface. For a negatively charged membrane and a positively charged peptide, the peptide concentration, C_M, at the interface is considerably larger than the bulk equilibrium concentration, c_{eq}:

$$C_M = C_{eq}\exp(-\langle z\rangle\psi_0 F_0/RT) \quad (5)$$

The binding process proper is characterized by the transition of the peptide from the layer of concentration C_M into the membrane phase. The term "binding" is, however, rather loosely defined since no specific binding sites (such as the phosphate group) are singled out in the present model. Instead, binding is considered simply as a restriction of dimensionality (Kim et al., 1991; Mosior & McLaughlin, 1991, 1992). In solution the ligand is free to undergo a three-dimensional walk; however, once bound to the membrane the motion of the ligand is restricted to a two-dimensional diffusion. Experimental evidence for a rapid diffusion of the peptide along the membrane surface has been provided by deuterium NMR (Beschiaschvili & Seelig, 1991).

The equilibrium between the plane of binding and the interfacial aqueous layer is described by a simple partition equilibrium according to

$$X_b = K_p C_M \quad (6)$$

where X_b denotes the molar amount of peptide bound per mole lipid and K_p is the partition constant (= *intrinsic* binding constant). More sophisticated versions of the law of mass action are conceivable, but eq 6 was found to provide a satisfactory fit to the data available at present. The measured heat of reaction, Δh, is related to the amount of bound peptide according to

$$\Delta h = \Delta H X_b C_L^0 V_{cell} \quad (7)$$

where ΔH is the molar heat of binding, C_L^0 is the lipid concentration in the cell (only the outer layer of the lipid vesicles is considered), and V_{cell} is the volume of the calorimeter cell. By combining eqs 4–7, it is possible to quantitatively analyze the calorimetric binding isotherms taking into account, in addition, that (i) the binding of peptide changes the membrane surface charge density σ and (ii) Na^+ ions bind to PG membranes with a binding constant of $K_{Na^+} = 0.6$ M^{-1} (Lau et al., 1981; Macdonald & Seelig, 1987).

The numerical simulation allows for three free parameters, namely, K_p, $\langle z\rangle$, and ΔH. However, the choice of the latter two is distinctly narrowed for the following reasons. The effective charge of the peptide in solution is $\langle z\rangle = 1.4$. The

Table III: Thermodynamic Parameters of SMS 201-995 Binding As Derived from Calorimetric Titration of Lipid Vesicles into Peptide Solutions

membrane POPC/POPG (mol %/mol %)	vesicle size d (nm)	pH	buffer: 0.1 M NaCl plus	$\langle z \rangle$	K_p (M^{-1})	ΔG (kcal/mol)	ΔH (kcal/mol)
75/25	30 (son)[a]	6.25	10 mM PO_4^-	1.9	90 ± 10	−5.1	−7.3 ± 0.2
75/25	30 (son)	7.4	10 mM PO_4^-	1.3	90 ± 10	−5.1	−12.0 ± 0.3
75/25	30 (son)	7.4	10 mM Tris	1.3	100 ± 10	−5.1	−6.3 ± 0.3
75/25	400	7.4	10 mM Tris	1.3	33[b]	−4.5[b]	+0.8 ± 0.1
75/25	planar	7.4	10 mM Tris	1.3	36 ± 4[c]	−4.5[c]	nd[d]
100/0	30 (son)	7.4	10 mM Tris	1.3	170 ± 20	−5.4	−6.8 ± 0.2
100/0	400	7.4	10 mM Tris	1.3	110 ● 10	−5.2	+0.7 ± 0.1

[a] Sonified. [b] Determined by physical separation of membrane and peptide phase. [c] Determined by centrifugation and ζ-potential measurements (Beschiaschvili & Seelig, 1990). [d] Not determined.

degree of binding X_b is small in the present experiments, and $\langle z \rangle$ hence remains close to its solution value, in spite of distinct pK shifts at the membrane surface. In agreement with previous monolayer, ζ-potential, and deuterium NMR experiments (Beschiaschvili & Seelig, 1990a, 1991), we find that $\langle z \rangle \simeq 1.3 \pm 0.1$ yields a satisfactory explanation of all calorimetric curves.[2] A similar restriction is found for ΔH. By titrating the peptide into a large excess of lipid, almost all peptide is bound to the membrane. Without resorting to theoretical models, it is then possible to evaluate ΔH directly from the measured heat of reaction with 95% (mixed POPG/POPC) to 70% accuracy (POPC vesicles). Hence only K_p is truly a "free" parameter. The results derived from the calorimetric titration curves are summarized in Table III.

Intrinsic Binding Enthalpies. The binding of TNS at pH 7.4 and that of SMS 201-995 at pH 6.25 to sonified vesicles gives rise to an exothermic reaction which is, however, independent of the buffer composition (cf. Tables I and II). Hence, the binding of these two molecules in their almost fully charged form is not accompanied by a change in protonation. The measured binding enthalpies of −9.4 kcal/mol for TNS and −7.3 kcal/mol for SMS 201-995 thus reflect the true (intrinsic) binding enthalpies, denoted ΔH_1 in the following.

Next, we consider the same peptide binding reaction at pH 7.4. For pure POPC vesicles, Figures 4 and 5 demonstrate a *release* of $n = 0.24$ protons into the buffer medium, and the intrinsic binding enthalpy ΔH_1 is thus masked by additional protonation and deprotonation steps. For a thermodynamic analysis, the binding reaction can be divided into three steps

$$P/PH^+ \text{ (water)} \rightarrow P/PH^+ \text{ (membrane)}; \quad \Delta H_1$$

$$PH^+ \text{ (membrane)} \rightarrow P \text{ (membrane)} + H^+_{aq}; \quad \Delta H_2$$

$$H^+_{aq} + B^-_{aq} \rightarrow HB_{aq}; \quad -\Delta H_{diss}$$

with the overall reaction

$$PH^+ \text{ (water)} + B^- \rightarrow P \text{ (membrane)} + HB; \quad \Delta H$$

First, the peptide with its amino-terminal either charged (PH^+) or noncharged (P) enters the membrane. The corresponding binding enthalpy ΔH_1 is assumed to be identical for both species. Secondly, the amino group loses its proton (dissociation enthalpy ΔH_2). Finally, the released proton associates with the buffer (reaction enthalpy $-\Delta H_{diss}$). In calculating the total enthalpy balance, we note that, for each mole of peptide bound (protonated plus deprotonated species), only a mole fraction of n protons ($n < 1$) is released:

$$\Delta H = \Delta H_1 + n\Delta H_2 - n\Delta H_{diss} \quad (8)$$

Since ΔH denotes the measured heat of reaction, a comparison of eq 8 with the experimental result of eq 3 yields

$$-4.2 = \Delta H_1 + 0.237\Delta H_2 \text{ (kcal/mol)} \quad (9)$$

where ΔH_1 and ΔH_2 are still unknown. However, a similar relationship can be derived for the binding of the peptide to negatively charged POPG/POPC vesicles. Both equations together then allow the evaluation of ΔH_1 and ΔH_2.

As evidenced by Figure 5, the binding of the peptide to mixed POPC/POPG membranes leads to an *uptake* of protons which is in contrast to the *deprotonation* observed for neutral membranes. As the dissociation enthalpy of the buffer increases, the measured heat of reaction becomes less and less exothermic. The thermodynamic analysis can again be divided into three steps

$$P/PH^+ \text{ (water)} \rightarrow P/PH^+ \text{ (membrane)}; \quad \Delta H_1$$

$$P \text{ (membrane)} + H^+ \rightarrow PH^+ \text{ (membrane)}; \quad -\Delta H_2$$

$$HB \rightarrow H^+ + B^-; \quad \Delta H_{diss}$$

with the overall reaction

$$P \text{ (water)} + HB \rightarrow PH^+ \text{ (membrane)} + B^-; \quad \Delta H$$

The peptide (PH^+ and P) binds to the membrane (ΔH_1), the noncharged species is protonated ($-\Delta H_2$), and the proton concentration is brought back to its initial value by buffer dissociation. Again, for each mole of peptide bound, only the fractional amount n participates in the protonation step, hence

$$\Delta H = -n\Delta H_2 + \Delta H_1 + n\Delta H_{diss} \quad (10)$$

By comparison of eq 10 with the experimental result given by eq 1, we obtain

$$-13.4 = \Delta H_1 - 0.626\Delta H_2 \quad (11)$$

Combining eqs 9 and 11 finally leads to $\Delta H_1 = -6.7$ kcal/mol for the association of the charged or noncharged peptide with sonified membrane vesicles and to $\Delta H_2 = +10.7$ kcal/mol for the dissociation enthalpy of the terminal amino group of SMS 201-995. The latter result is in excellent agreement with dissociation enthalpies of 11 ± 2 kcal measured for N-terminal amino groups of proteins in solution [cf. Martin (1964), p

[2] Previous SMS 201-995 binding isotherms (obtained from a centrifugation assay and ζ-potential measurements) were explained by assuming an average charge of $\langle z \rangle = 1.3$, but we were not aware of the pK value of pK = 7.2 of the terminal amino group (Beschiaschvili & Seelig, 1990a). We erroneously assumed that the total peptide charge was +2 of which only 65% was "membrane-visible" for steric reasons. This argument no longer holds true in the light of the present pK determination.

79]. Likewise, the binding enthalpy of $\Delta H = -6.7$ kcal/mol is in good agreement with the calorimetric measurement at pH 6.25, yielding $\Delta H = -7.3$ kcal/mol (again obtained for *sonified* vesicles; cf. Table II).

The above analysis leads to an average enthalpy ΔH_1 for the protonated and nonprotonated peptide species. However, since the binding is mainly hydrophobic in nature, the difference between the individual ΔH_1 values should not be large. Likewise, differences in the association enthalpy of the peptide with pure POPC and mixed POPC/POPG (75/25 mol/mol) membranes are ignored. These differences are small as demonstrated by Figure 6.

Finally, we can also calculate the peptide binding enthalpy for extruded POPC/POPG (75/25 mol/mol) vesicles with a diameter of $d \simeq 100$ nm. We assume that the dissociation enthalpy $\Delta H_2 = 10.7$ kcal/mol is independent of the vesicle size. Insertion of this value into eq 10 and comparison with the experimental result of eq 2 leads to $\Delta H_1 \simeq 0$ kcal/mol at pH 7.4. Again this result is in good agreement with the direct measurement at pH 6.25 leading to $\Delta H = -1.4 \pm 0.3$ kcal/mol for 100-nm vesicles (cf. Table II).

In conclusion, the thermodynamic analysis leads to the following results: (i) The dissociation enthalpy of the SMS 201-995 Phe^1 amino group is $\Delta H_2 = +10.7$ kcal/mol, which is close to the dissociation enthalpy of Tris buffer. Calorimetric titrations in Tris buffer thus reflect rather closely the true association enthalpy of SMS 201-995 due to the compensatory mechanism of the two amino groups. (ii) The *intrinsic* enthalpy change for the binding of the somatostatin analog to *sonified* lipid vesicles is $\Delta H_1 \simeq -7.0 \pm 0.3$ kcal/mol, independent of pH. (iii) The binding enthalpy for SMS 201-995 with vesicles of larger diameter ($d \leq 100$ nm) is $\Delta H_1 \simeq -1$ to $+1$ kcal/mol.

Membrane-Induced pK Shifts. Titration calorimetry is a rather direct and elegant method to study reactions which are accompanied by a release or an absorption of protons (Flogel & Biltonen, 1975; Biltonen & Langerman, 1979; Morin & Freire, 1991). In simple systems, a plot of the observed ΔH values versus the buffer dissociation enthalpies, ΔH_{diss}, yields a straight line with the slope determined by the number of protons released or adsorbed during the reaction process. For zwitterionic POPC vesicles, the experimental results can be explained by a *release* of $n = 0.24$ protons into the buffer medium. While the charge of the peptide is $\langle z \rangle = 1.4$ in pure buffer, this value therefore is reduced to $\langle z \rangle = 1.15$ for the membrane-bound peptide. At low degrees of peptide binding, the apparent pK_M of the membrane bound peptide can be calculated according to

$$pK_M = pH - \log [(z_{pH^+} - \langle z \rangle)/(\langle z \rangle - z_p)] \qquad (12)$$

(pH is the bulk pH 7.4, $z_{pH^+} = 2$ is the fully protonated peptide, and $z_p = +1$ is the peptide with the amino-terminal deprotonated). Using $\langle z \rangle = 1.15$, the pK_M is found to be $pK_M = 6.65$, which is equivalent to a pK shift of $\Delta pK = -0.55$ referred to a $pK = 7.2$ in bulk solution. The driving force for the proton release in the neutral POPC membrane appears to be the lower dielectric constant at the membrane interface which destabilizes the terminal NH_3^+ group.

A completely different situation is encountered for charged POPC/POPG membranes. The surface potential of a POPG/POPC (25/75 mol/mol) membrane can be calculated via the Gouy–Chapman theory as $\psi_0 = -47$ mV (lipid area 68 Å^2, 0.10 M NaCl, 10 mM Tris buffer; Na^+ binding to PG taken into account with a binding constant of $K_{Na} = 0.6$ M^{-1}). Due to this negative surface potential, the proton concentration increases from its bulk value of pH 7.4 to pH 6.6 at the membrane surface. The effective charge of the peptide will increase concomitantly from $\langle z \rangle = 1.4$ to $\langle z \rangle = 1.8$. The pH shift at the negatively charged membrane surface thus predicts an uptake of $n = 0.4$ protons per bound peptide molecule. The experimental result obtained for large vesicles (eq 2) is $n = 0.38$ in agreement with the theoretical analysis. The apparent pK can be calculated as $pK = 8.0$ and the pK shift is $\Delta pK = 0.8$.

Since the pK shift of charged POPC/POPG vesicles can be explained exclusively on the basis of a pH gradient at the membrane surface, this finding implies that the polarity-induced pK shift as observed for neutral POPC membranes ($\Delta pK = -0.55$) does not play a role for mixed POPC/POPG membranes. Apparently, the terminal amino group of the peptide remains close to the negatively charged POPG headgroups, even though the interaction of the two groups is probably rather weak.

Polarity- and pH-induced pK shifts at membrane surfaces have been discussed extensively for potential-sensitive dyes (Fernandez & Fromherz, 1977; Fromherz, 1989) and potential sensitive spin labels (Miyazaki et al., 1992; Cevc & Marsh, 1987), but little is known for proteins. The polarity-induced pK shifts for dyes or spin labels are typically about $|\Delta pK| \approx 1$ and thus larger than the polarity induced pK shift for SMS 201-995 in neutral POPC membranes. Probably the N-terminal amino group sinks less deeply into the membrane phase than the carboxylic acids or amines employed in earlier studies.

Electrostatic pK shifts depend critically on the surface charge of the membrane and may reach values of $|\Delta pK| \approx 1$–2 for dyes and spin labels. An electrostatic pK shift of a peptide has been observed for the terminal amino group of substance P incorporated into SDS micelles (Wolley & Deber, 1987) and also for substance P micelles (Seelig, 1990). The electrostatic pK shift was $\Delta pK \approx 1$ toward larger pK values, in agreement with the present results on the somatostatin analog where a $\Delta pK \simeq 0.8$ was observed.

Membrane–Peptide Thermodynamics. Let us first consider the binding of fully charged TNS (pH 7.4) and SMS 201-995 (pH 6.25). While TNS ($z = -1$) and SMS 201-995 ($\langle z \rangle = 1.9$) are oppositely charged, both binding reactions are clearly exothermic with $\Delta H = -9.4$ kcal/mol for TNS and $\Delta H = -7.3$ kcal/mol for the somatostatin analog. The corresponding partition constants are 5000 M^{-1} for TNS (McLaughlin & Harary, 1976) and 90 M^{-1} for the peptide (Figure 2). The free energy of binding can be evaluated according to

$$-\Delta G = RT \ln (55.5 K_p) \qquad (13)$$

where the factor 55.5 corrects for the cratic contribution [cf. Cantor and Schmimmel (1981)]. The free energies are found to be -7.4 kcal/mol for TNS and -5.1 kcal/mol for SMS 901-995. For sonified vesicles at pH 6.25, the binding of the almost fully charged peptide is thus completely enthalpy driven, indicating a "nonclassical" hydrophobic interaction as has been found previously for TNS (Huang & Charley, 1972) and other small amphiphilic molecules (Bäuerle & Seelig, 1991; Seelig & Ganz, 1991).

Table III then provides a synopsis of the thermodynamic parameters as derived from titration calorimetry of lipid vesicles into peptide solutions (Figure 2). First, it should be noted that the intrinsic binding constant K_p is independent of pH and buffer composition and amounts to $K \approx 100$ M^{-1} for sonified POPC/POPG (75/25 mol/mol) membranes. K increases to 170 M^{-1} for sonified POPC membranes and decreases to $K \approx 35$ M^{-1} for large unilamellar vesicles or

coarse liposomes. The Gibbs free energy as calculated according to eq 13 is thus rather constant for all membrane systems studied ($\Delta G = -4.4$ to -5.4 kcal/mol). In particular, the difference between small sonified POPC/POPG vesicles (75/25 mol/mol; $\Delta G = -5.1$ kcal/mol) and large extruded vesicles ($\Delta G = -4.6$ kcal/mol) is only $\delta\Delta G = +0.5$ kcal/mol. At the same time, the intrinsic binding enthalpy (corrected for pK shifts) undergoes quite a dramatic change from $\Delta H \simeq -7$ kcal/mol for vesicles with $d \simeq 30$ nm to $\Delta H \simeq +1$ kcal/mol for vesicles with $d \simeq 400$ nm, i.e., $\delta\Delta H \simeq +8$ kcal/mol. The approximate constancy of ΔG together with the pronounced variation of ΔH necessitates an equally strong variation of $T\Delta S$. While the peptide binding is *enthalpy*-driven for *sonified* vesicles, it becomes *entropy*-driven for large vesicles.

A possible explanation of this enthalpy–entropy compensation mechanism follows from a consideration of the thermoelastic stress of a constrained lipid bilayer. The bilayer membrane has a large resilience of volume, and its isothermal compressibility coefficient $\chi = -(1/V)(\partial V/\partial p)_T$ is small and comparable to that of other organic liquids ($\chi \simeq 10^{-5}$ atm^{-1}). In contrast, it has a much smaller resilience of area, i.e., bilayers are very "soft" condensed surfaces (Bloom et al., 1991) which are easy to compress or expand. The variation of the internal energy U and the entropy S with the surface area A follows from standard thermodynamic considerations:

$$\left(\frac{\partial U}{\partial A}\right)_T = T\left(\frac{\partial S}{\partial A}\right)_T - \pi = T\left(\frac{\partial \pi}{\partial T}\right)_A - \pi = T\frac{\alpha}{\chi} - \pi = \pi_i \quad (14)$$

$$T\left(\frac{\partial S}{\partial A}\right)_T = T\left(\frac{\partial \pi}{\partial T}\right)_A = T\frac{\alpha}{\chi} \quad (15)$$

$$\left(\frac{\partial F}{\partial A}\right)_T = \left(\frac{\partial U}{\partial A}\right)_T - T\left(\frac{\partial S}{\partial A}\right)_T = -\pi \quad (16)$$

Here $\alpha = (1/A)(\partial A/\partial T)_\pi$ defines the area expansivity at constant membrane tension π, $\chi = -(1/A)(\partial A/\partial \pi)_T$ is the isothermal area compressibility, and F is the Helmholtz free energy. It should be noted that π is the bilayer lateral tension and not the external pressure. Elastic measurements on bilayer membrane vesicles [reviewed in Bloom et al. (1991)] and of monolayers compressed to the bilayer equivalence pressure (Davies & Jones, 1992) yield $T\alpha/\chi \approx 0.3 - 0.4$ J/m^2 = 72 – 97 mcal/m^2 at room temperature, which is about one order of magnitude larger than the bilayer tension $\pi \simeq 0.03$ J/m^2 = 7.2 mcal/m^2. The above equations demonstrate that large energy changes $\delta U = (T\alpha/\chi - \pi)\delta A$ and entropy changes $T\delta S = (T\alpha/\chi)\delta A$ can be associated with an area variation δA. The dominating term $T\alpha/\chi$ cancels, however, if one considers the variation of the free energy $\delta F = -\pi\delta A$. In other words, the increase in the surface area of the lipid membrane is accompanied by a distinct absorption of heat (= δU), leading to an almost equally large entropy increase (= $T\delta S$). The latter is suggested to be due to a disordering of the hydrocarbon chains, which means that the elastic tension of the bilayer has a purely entropic origin.

For the problem at hand, we make three assumptions: (i) the incorporation of foreign molecules such as TNS or SMS 201-995 induces a small disordering of the hydrocarbon chains leading to an area increase δA, (ii) the area expansion is roughly proportional to the size of the penetrating molecule, and (iii) the *internal* tension, defined above (eq 14) as $\pi_i = T\alpha/\chi - \pi$, is larger for well-packed planar bilayers (strong cohesive forces) than for sonified vesicles, i.e., $\pi_{i,p} > \pi_{i,v}$, as suggested by geometric packing constraints. For the sake of the argument, we assume that the internal tension π_i is 20% larger for planar bilayers than for lipid vesicles, the difference being $\Delta\pi_i \approx 17$ mcal/m^2. This value is suggested by the area dependence of π_i in monolayer experiments [Davies and Jones (1992), Table I) but is not too critical for the following discussion.

In applying this model to TNS binding, we recall that insertion of TNS into large unilamellar vesicles ($d = 100$ nm) requires more energy (δU_p) than insertion into sonified vesicles (δU_v). With the equality

$$\delta U_p - \delta U_v = \Delta\pi_i \delta A_{TNS} \quad (17)$$

and with $\delta U_p - \delta U_v \simeq 2.0$ kcal/mol (calorimetric result) and $\Delta\pi_i = 17$ mcal/m^2, we estimate a TNS-induced increase in the lipid surface area of $\delta A_{TNS} = 1.2 \times 10^5$ m^2 per mole of TNS. We note that the degree of TNS binding is always smaller than $X_b = 0.1$ (moles of TNS/mole of lipid) and that the total surface area of 1 mol of bilayer lipid is 4.1×10^5 m^2. Under these conditions, i.e., 1 mol of TNS/10 mol of lipid, the binding of 1 mol of TNS would produce a 3% change in the total lipid area.

SMS 201-995 (MW 1019.3) has a larger surface area than TNS (MW 313.8). We assume that the SMS-induced increase in the lipid surface area is $\delta A_{SMS} \simeq 3.2\delta A_{TNS}$ as suggested by the ratio of the molecular weights. Insertion into eq 17 predicts $\delta U_p - \delta U_v \approx 6.4$ kcal/mol, which is close to the experimental value of +8 kcal/mol.

We have recently measured the binding enthalpy of melittin (MW 2846) to POPC/POPG vesicles with titration calorimetry. The enthalpy difference between extruded vesicles ($d \simeq 100$ nm) and sonified vesicles was found to be $\delta\Delta H = \delta U_p - \delta U_v \simeq 19$ kcal/mol, which is distinctly larger than the result obtained for SMS 201-995 (Beschiaschvili and Seelig, manuscript in preparation). Using again the crude assumption that the increase in lipid surface area scales with the size of the penetrating molecule, we estimate $\delta A_{melittin}/\delta A_{TNS} \simeq 2846/313$ and predict a difference in the binding enthalpies of 18.2 kcal/mol, in good agreement with the experiment.

The pronounced calorimetric differences between sonified lipid vesicles and extruded vesicles of large diameter suggest a new model for the binding of amphiphatic peptides and other amphiphatic molecules to membranes. For sonified vesicles the binding is exclusively enthalpy-driven. The water molecules released from the peptide appear to be rebound at the membrane surface, leading to an almost zero entropy contribution. As the membrane curvature decreases, the packing constraints of the lipids are relaxed and the internal tension π_i increases. Hence, considerably more energy is required to insert a foreign molecule between the lipids of a planar membrane, compensating in part or totally the gain in van der Waals energy. At the same time, the peptide-induced area increase entails an entropy increase such that $T\delta S \sim \delta U$. For large molecules, the binding to the membrane may become completely entropy-driven. However, in contrast to the classical hydrophobic effect, the molecular origin of this entropy increase is not a release of the water molecule from the protein surface but an increase in the hydrocarbon chain disorder.

The entropy-producing potential of the lipid matrix and its role in membrane binding equilibria has not been appreciated so far. The size of this entropy source is considerable as is obvious from a comparison of the solid-to-fluid transition of simple hydrocarbons with the gel-to-liquid-crystal transition of phospholipid bilayers [a review of thermodynamic data may be found in Seelig (1981) or Cevc and Marsh (1987)].

From the chain length dependence of the transition enthalpies, it was found that the melting of pure fatty acids or solid paraffins required an incremental transition enthalpy of $\Delta H_{increment} \approx 1.0$ kcal/mol of CH_2 and was accompanied by an entropy production of $\Delta S_{increment} \approx 2.6$ cal/deg·mol of CH_2. In contrast, the gel-to-liquid-crystal transition of disaturated phospholipids (with different headgroups) required a smaller enthalpy of $\Delta H_{increment} = 0.5$ kcal/mol of CH_2 and entropy $\Delta S_{increment} = 1.3 - 1.6$ cal/deg·mol of CH_2. Since $\Delta H_{increment}$ and $\Delta S_{increment}$ are clearly lower for phospholipids than for paraffins, the hydrocarbon chains in a liquid-crystalline bilayer are more restricted in their conformational freedom than in a true liquid paraffin (Phillips et al., 1969). Hence, we consider the difference between the two situations as the maximum "entropy potential" of the lipid membrane. Since the average chain length of phospholipids is 16–20 carbon atoms and since each phospholipid carries two fatty acyl chains, this "entropy potential" can be quantified as $T\Delta S \simeq 298(2 \times 16 \times 1) \simeq 9500$ cal/mol of phospholipid or ~ 23 mcal/m^2 assuming a lipid surface area of 68 Å^2. Since this value corresponds to the difference in entropy between a liquid-crystalline phospholipid bilayer with semifluid hydrocarbon chains and a fully fluid isotropic hydrocarbon, it must be considered as an upper limit. It may be compared with a value of 17 mcal/m^2 which was used as the difference in internal pressure π_i between the sonified vesicles and the more ordered planar bilayer.

Finally, we briefly consider the changes in the free energy with vesicle size. Even though δF is only of the order of 10% of the δU or $T\delta S$ term, systematic variations with increasing internal pressure are obvious. Inspection of Table III demonstrates that the free energy of SMS 201-995 binding becomes less negative by about 0.5 kcal/mol for large vesicles, corresponding to a decrease in the binding constant by a factor of 3–4. A similar result was observed for melittin where the binding constant of sonified vesicles was larger by a factor of 10–20 (= 1.5 kcal/mol) than that of planar membranes (Kuchinka & Seelig, 1989; Beschiaschvili & Seelig, 1990b). Furthermore, the partition coefficients of benzene and hexane into phospholipid membranes have been measured as a function of the packing density of the lipids (de Young & Dill, 1989, 1990). With increasing surface density of the lipids, the partition constant decreased smoothly (by at most a factor of 10), which is in agreement with the results obtained for peptides. In the above model, δF is the difference between two large numbers ($\delta F = \delta U - T\delta S$), and a molecular interpretation of δF in terms of either the van der Waals energy or the lipid disorder appears not to be warranted.

CONCLUSIONS

Using high-sensitivity titration calorimetry and a conformationally constrained peptide, we have demonstrated that the binding of the peptide to sonified lipid vesicles is a completely enthalpy-driven reaction. The classical hydrophobic effect with a release of water molecules from the associating surfaces appears to play no role for sonified vesicles.

A small increase in vesicle size leads to a decrease in the enthalpic component and an increase in the entropic contribution. For large bilayer vesicles, the reaction becomes entropy-driven. Though the possibility of a completely different binding mechanism for extruded vesicles, involving the classical hydrophobic effect, cannot be excluded, we prefer to assume a smooth variation of the thermodynamic state functions U, S, and F with increasing internal bilayer pressure π_i. While U and S increase strongly with π_i, the variation of F remains small explaining the observed enthalpy–entropy compensation. Under these circumstances, the driving force for the binding reaction appears to be the disordering of the lipid chains. At low degrees of peptide binding, only small changes per lipid acyl chain are necessary to generate the required entropy increase.

The binding reaction of SMS 201-995 was found to be accompanied by a pK shift which was dependent on the membrane composition. Since many terminal amino groups have a pK value of p$K \sim 7$–8 (Martin, 1964), a peptide pK shift is predicted to be a common phenomenon in peptide–membrane partition equilibria.

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