Specific Binding of Ro 09-0198 (Cinnamycin) to Phosphatidylethanolamine: A Thermodynamic Analysis†

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ABSTRACT: Ro 09-0198 (cinnamycin) is a tetracyclic peptide antibiotic that is used to monitor the transbilayer movement of phosphatidylethanolamine (PE) in biological membranes during cell division and apoptosis. The molecule is one of the very rare examples where a small peptide binds specifically to a particular lipid. In model membranes and biological membranes containing phosphatidylethanolamine, Ro 09-0198 forms a 1:1 complex with this lipid. We have measured the thermodynamic parameters of complex formation with high sensitivity isothermal titration calorimetry and have investigated the structural consequences with deuterium and phosphorus solid-state NMR. Complex formation is characterized by a large binding constant, K_0, of 10^7 to 10^8 M^{-1}, depending on the experimental conditions. The reaction enthalpy, $\Delta H°$, varies between zero at 10 °C to strongly exothermic −10 kcal/mol at 50 °C. For large vesicles with a diameter of ∼100 nm, $\Delta H°$ decreases linearly with temperature and the molar heat capacity of complex formation can be evaluated as $\Delta C_p° = -245$ cal/mol, indicating a hydrophobic binding mechanism. The free energy of binding is $\Delta G° = -10.5$ kcal/mol and shows only little temperature dependence. The constancy of $\Delta G°$ together with the distinct temperature-dependence of $\Delta H°$ provide evidence for an entropy−enthalpy compensation mechanism: at 10 °C, complex formation is completely entropy-driven, at 50 °C it is enthalpy-driven. Varying the PE fatty acid chain-length between 6 and 18 carbon atoms produces similar binding constants and $\Delta H°$ values. Addition of Ro 09-0198 to PE containing bilayers eliminates the typical bilayer structure and produces ^{2}H- and ^{31}P-NMR spectra characteristic of slow isotropic tumbling. This reorganization of the lipid matrix is not limited to PE but also includes other lipids.

Many amphiphilic or hydrophobic peptides bind unspecifically to biological membranes by a physical adsorption mechanism, penetrating more or less deeply into the hydrophobic core of the lipid bilayer. In contrast, specific phospholipid−peptide interactions are rare. One of the very few known exceptions is provided by the 19-amino acid tetracyclic peptide Ro 09-0198 (cinnamycin) which forms a tight equimolar complex with phosphatidylethanolamine (*1*, *2*). Ro 09-0198 has been isolated from *Streptoverticillium griseoverticillatum* (*3*) and is identical to cinnamycin isolated from *Streptomyces cinnamoneus* (*4*). The chemical structure of the peptide has been determined by NMR[1] (*5*, *6*) and by chemical methods (*7*) and is given in Figure 1. Posttranslational side chain modifications result in unusual amino acids typical for the whole group of lantibiotics (*4*). The solution structure of the Ro 09-0198 complex with lyso-phosphatidylethanolamine was determined in DMSO by ^{1}H NMR (*8*).

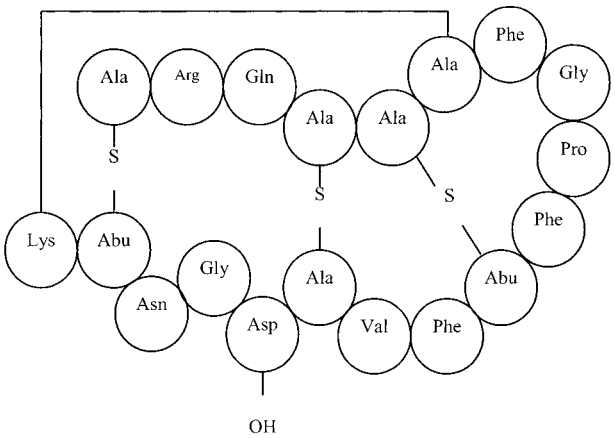

FIGURE 1: Primary structure of the lantibiotic Ro 09-0198 (cinnamycin). Ala−S−Ala = lanthionine; Abu−S−Ala = 3-methyllanthionine.

Ro 09-0198 has found important applications as a novel probe for studying the transbilayer movement of phosphatidylethanolamine (*9*). In eukaryotic plasma membranes, amino phospholipids such as phosphatidylethanolamine and phosphatidylserine are usually located on the inner leaflet. However, several processes of cell activation can induce a rapid flip-flop of phospholipids leading to an exposure of phosphatidylethanolamine on the membrane outside where it then becomes detectable with fluorescence-labeled Ro 09-0198 (*10*).

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[1] Abbreviations: ITC, isothermal titration calorimetry; PE, phosphatidylethanolamine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; LUV, large unilamellar vesicles of ∼100 nm diameter; SUV, small unilamellar vesicles of ∼30 nm diameter; NMR, nuclear magnetic resonance; [9′,10′-$^2H_2$]POPE, 1-palmitoyl-2-[9′,10′-$^2H_2$]oleoyl-*sn*-glycero-3-phosphoethanolamine.

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The specific interaction of Ro 09-0198 with phosphatidylethanolamine has been described at a qualitative level so far (*1*, *2*). No thermodynamic data are available in the literature. We have therefore measured the complex formation between Ro 09-0198 and different phosphatidylethanolamines with high sensitivity isothermal titration calorimetry and have determined the stability constant of complex formation, K_0, the reaction enthalpy, $\Delta H°$, and the free energy of binding, $\Delta G°$, in the temperature interval of 10−50 °C. Using deuterated phosphatidylethanolamine in combination with solid-state deuterium NMR, we have furthermore investigated the structural consequences of the PE/peptide complex in the membrane.

MATERIALS AND METHODS

Materials. 1,2-Dihexanoyl-*sn*-glycero-3-phosphoethanolamine (C_6-PE), 1,2-dioctanoyl-*sn*-glycero-3-phosphoethanolamine (C_8-PE), 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine (C_{12}-PE), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (C_{14}-PE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were purchased from Avanti Lipids, Alabaster, AL. Ro 09-0198 was a generous gift of Dr. R. Imhof, Hoffmann-La Roche Ltd., Basel, Switzerland. 1-Palmitoyl-2-[9′,10′-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine was synthesized as described by Seelig and Waespe-Sarcevic (*11*). The corresponding phosphatidylethanolamine, denoted [9′,10′-$^2H_2$]POPE in the following, was prepared with transphosphatidylation by phospholipase D (*12*).

Preparation of Lipid Vesicles. Vesicles were prepared as POPC/POPE mixtures (10:1 wt/wt) at a final lipid concentration of 10 mg/mL. To this purpose, stock solutions of POPC and POPE in methanol/chloroform were prepared. The appropriate amount of POPC was filled into a 5-mL round-bottomed flask and was dried down under nitrogen. Residual solvent was removed by applying high vacuum. The amount of POPC was controlled by weighing. Next, POPE dissolved in chloroform/methanol was added, and the procedure was repeated. A total of 2 mL of buffer (10 mM Tris HCl, and 0.1 M NaCl, pH 7.4) was added to the dry film, and the suspension was extensively vortexed. Next, small unilamellar vesicles (SUVs) were prepared by sonication of the lipid suspension for 20−40 min until an almost clear solution was obtained (at 4 °C under a nitrogen atmosphere). Metal debris of the tianium tip was removed by centrifugation in an Eppendorf centrifuge (10 min at 3000 rpm). Sonication produces vesicles with an average diameter of ∼30 nm.

For the preparation of 100 nm large unilamellar vesicles (LUVs), the lipid suspension was first subjected to at least four freeze−thaw cycles and was then extruded 12 times through two stacked polycarbonate filters of 0.1 μm pore size (Nucleopore, Whatman, UK) (*13*).

High-Sensitivity Titration Calorimetry. Isothermal titration calorimetry (ITC) was performed with a Microcal VP-ITC titration calorimeter [Microcal, Northampton, MA (*14*)]. Solutions were degassed under vacuum before use. The calorimeter was calibrated electrically. Injection volumes varied between 3 and 20 μL. Peptide concentrations varied between 40 and 200 μM. The calorimeter cell had a reaction volume of 1.4037 mL.

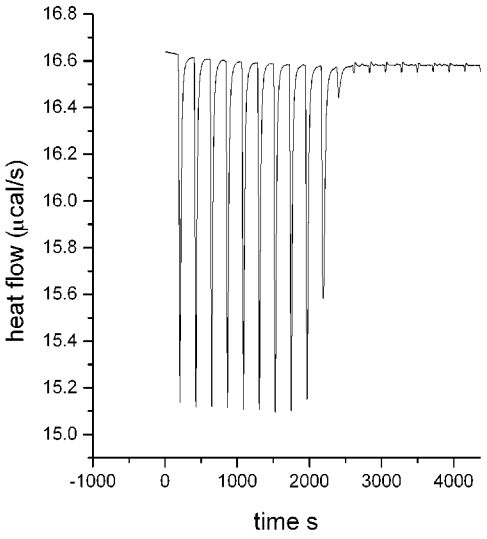

FIGURE 2: Isothermal titration calorimetry. The lantibiotic Ro 09-0198 (cinnamycin) is titrated with large unilamellar vesicles (LUVs) containing phosphatidylethanolamine. The peptide concentration in the reaction cell is 40 μM. The phospholipid vesicles (∼100 nm diameter) are composed of POPC/POPE 10:1 wt/wt. The total phospholipid concentration is ∼10 mg/mL, and the concentration of PE alone is 1.21 mM. Each peak corresponds to the injection of 8 μL of lipid suspension into the reaction cell (V_{cell} = 1.4037 mL). Buffer: 10 mM Tris, 100 mM NaCl. Temperature 45 °C.

NMR Spectroscopy. All spectra were acquired on a Bruker DRX400 spectrometer operating at a frequency of 61.4 MHz for ^{2}H NMR and 162 MHz for ^{31}P NMR. For the ^{2}H NMR measurements, a quadrupole echo sequence (*15*) was employed with a full-phase cycling. The 90° pulse duration was 5.0 μs, the interpulse delay was 40 μs, and the recycle delay was 250 ms (the ^{2}H longitudinal relaxation time was T_1 ∼ 16 ms at 30 °C). The spectral width was 64 kHz, and typically 5000 signal averaged accumulations were Fourrier transformed to yield a spectrum. The ^{31}P NMR spectra were recorded using a Hahn echo sequence (*16*) with broadband proton decoupling (WALTZ-16) and phase cycling. The 90° pulse duration was 3.0 μs, the interpulse delay was 40 μs, and the recycle delay was 5 s (the ^{31}P longitudinal relaxation time was T_1 ∼ 0.8 s at 30 °C). The spectral width was 32 kHz, and 200 transients were recorded for a spectrum.

RESULTS

Specific PE binding of Ro 09-0198 is illustrated in Figure 2 which displays the titration of a Ro 09-0198 solution (40 μM) with phospholipid vesicles containing phosphatidylethanolamine. The vesicles are composed of about 9 wt % POPE mixed with an excess of POPC (POPE/POPC 1:10 wt/wt). Large unilamellar vesicles (LUVs) of ∼100 nm diameter were prepared by extrusion through polycarbonate filters. Each addition of lipid to the peptide solution causes a distinct exothermic reaction with a constant reaction enthalpy, h_i, for the first 10 injections. At the 11th injection, the reaction enthalpy suddenly drops and is zero at all following injections. This titration pattern lends itself to a simple molecular interpretation. Initially, the peptide is much in excess over the added lipid. Upon injection of lipid vesicles the peptide molecules react immediately with the added PE (on the vesicle outside), and the concentration of free peptide in solution is reduced accordingly. After

11 injections, a sufficient amount of POPE is added to bind all available peptide and addition of further lipid has no effect.

A simple quantitative interpretation can also be given. The total heat measured is $\sum_1^{10} h_i = -496\ \mu$cal, the total amount of peptide in the sample cell is $n_P^\circ = 56.15$ nmol, and the reaction enthalpy is thus determined as $\Delta H^\circ = \sum h_i / n_P^\circ = -8.8$ kcal/mol peptide. The amount of phosphatidylethanolamine (POPE) added in the first 10 steps is, however, $n_L^\circ = 96.5$ nmol and is thus larger than the amount of available peptide. If a 1:1 complex is formed, only 58% of the total POPE appears to be available for peptide binding. This corresponds to the POPE content of the outer layer of the vesicle and demonstrates that under the conditions of the experiment Ro 09-0198 cannot cross the bilayer membrane.

A further proof for half-layer binding of Ro 09-0198 was provided by the inverse titration experiment (*17*) where the sample cell contained the lipid vesicles (~10 μM) and the injection syringe was filled with peptide solution (~100 μM). The exothermic reaction was found to drop to zero as soon as the added amount of peptide approached ~55% of the total POPE in the sample cell.

The two types of titration experiments therefore lead to three conclusions: (i) POPE and Ro 09-198 form a 1:1 complex, (ii) only PE on the vesicle outside is available for binding, and (iii) the binding reaction is strongly exothermic. The first two conclusions hold true for practically all titrations described in this study. In contrast, ΔH° was found to vary distinctly with temperature and even changed its sign at low temperatures. In addition, if the PE content of the POPC/POPE mixtures was larger than 20%, all PE became accessible to Ro 09-0198.

A quantitative description of the titration pattern (Figure 2) is possible in terms of a chemical complex formation according to $P + L \rightleftarrows PL$. Here P, L, and PL denote the peptide, the PE lipid, and the peptide/lipid 1:1 complex, respectively. The chemical equilibrium can be written as

$$\frac{C_{PL}}{C_P \cdot C_L} = \frac{C_{PL}}{(C_P^0 - C_{PL}) \cdot (C_L^0 - C_{PL})} = K_0 \qquad (1)$$

$$C_{PL} = \frac{1}{2}\left(C_L^0 + C_P^0 + \frac{1}{K_0}\right) - \frac{1}{2}\sqrt{\left(C_L^0 + C_P^0 + \frac{1}{K_0}\right)^2 - 4C_L^0 C_P^0} \qquad (2)$$

C_P^0 and C_L^0 are the total peptide and lipid concentrations, respectively, in the sample cell. In a lipid(syringe)-to-peptide-(calorimeter cell) titration experiment (e.g., Figure 2), C_L^0 increases in steps of δC_L^0, while the peptide concentration C_P^0 remains constant (except for small dilution effects). The total heat absorbed or released after i injections is

$$\sum_i h_i = \Delta H^\circ\ C_{PL}^{(i)} \cdot V_{cell} \qquad (3)$$

where ΔH° is the reaction enthalpy and V_{cell} is the volume of the reaction vessel. Combining eqs 2 and 3 (including dilution effects), it is possible to fit the experimental titration curve h_i vs N_{inj} (Figure 2) with the proper set of thermodynamic parameters ΔH° and K_0.

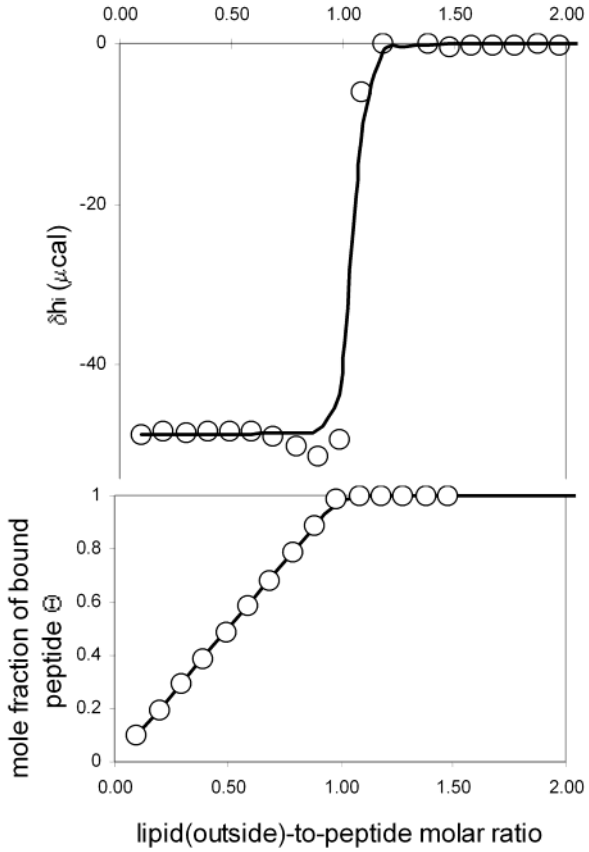

FIGURE 3: (A) Heats of reaction, h_i, as evaluated from the areas underneath the calorimeter tracings shown in Figure 2. h_i is plotted as a function of the phosphatidylethanolamine-to-peptide ratio. The latter is calculated on the basis of the phosphatidylethanolamine on the vesicle outside (theory: 50% of total PE; the fit requires 57%). The open symbols represent the experimental data. The solid line was calculated for a 1:1 PE/Ro 09−0198 complex with an association constant of $K_0 = 5 \times 10^7\ M^{-1}$ and an enthalpy of complex formation of $\Delta H^\circ = -8.8$ kcal/mol. (B) The mole fraction of bound Ro 09−0198 as a function of the PE (outside)-to-peptide ratio.

ΔH° can be determined with high precision from a given titration curve, but larger errors are associated with K_0. Figure 2 demonstrates a rather sharp transition from complex formation to a nonbinding situation. The steepness of the transition curve is directly related to K_0, and the accuracy of the evaluation depends on the number of data points in the transition region. Under the experimental conditions used to obtain Figure 2, typically 1−2 data points fall in the transition region. However, the transition can be broadened by reducing the concentration of the reactants. Inevitably, this also reduces the calorimetric signal. Optimum conditions for the evaluation of K_0 were found for the inverse titration experiment, that is, peptide(syringe)-to-lipid(calorimeter cell) titrations with lipid (PE) and Ro 09-0198 concentrations of about 10 and 100 μM, respectively. Under these conditions, the binding constants can be determined with an accuracy of 30%.

Figure 3 displays the analysis of the data given in Figure 2 in terms of the 1:1 complex formation model. The open symbols relate to the experimental data of Figure 2, the solid line is the theoretical analysis. Figure 3A shows the fit of the primary experimental data, h_i, versus the POPE-to-peptide ratio (POPE of vesicle outside only). In Figure 3B, the heats of reaction, h_i, are used to calculate the mole fraction of

Table 1: Thermodynamic Parameters of 1:1 Complex Formation

PE lipid	POPC/PE mole ratio	vesicle diameter nm	lipid availability factor f_L	ΔH_0 kcal/mol	K_0 10^7 M^{-1}	temp °C
POPE	10:1	100	0.56 ± 0.07	-4.5[a]	6 ± 3	28
POPE	87:1	100	0.5	-5.1	5 ± 1	28
POPE	10:1	30	0.7 ± 0.05	-6.5 ± 0.2	1.0 ± 0.2	28
POPE	8:3	30	1	-7.8	7	37
POPE	8:3	100	1	-7.1	7	37
C14 PE	10:1	100		-4.8 ± 1	4.3 ± 2	28
C12 PE	10:1	100		-5.3 ± 0.5	4.5 ± 2.5	28
C8 PE	54:1	100	0.52	-7.3 ± 1	6.8 ± 1.4	28
C6 PE	47:1	100	0.48	-5.7 ± 0.5	4.3 ± 1.8	28
POPC	no PE	30	~ 0.6	~ -1.0	$100-300$ M^{-1}	28

[a] The temperature dependence is linear for 100 nm vesicles with ΔH (kcal/mol) $= -0.2451$ T (°C) $+2.3$

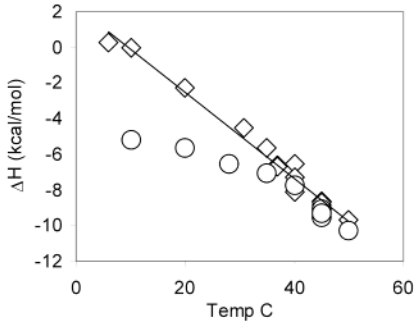

FIGURE 4: PE:Ro 09−0198 complex formation. The enthalpy of complex formation, ΔH°, is plotted vs the temperature for LUVs (◊) and SUVs (○). Vesicles were composed of POPC:POPE at a 10:1 wt/wt ratio.

bound peptide, Θ, according to

$$\Theta = \sum_{1}^{i} h_i / \sum_{1}^{N} h_i \qquad (4)$$

The binding constant, K_0, is $K_0 = 5 \times 10^7$ M^{-1} and the reaction enthalpy $\Delta H = -8.8$ kcal/mol (at 45 °C) for the data given in Figure 2.

A large number of titrations was performed, varying temperature, vesicle size, fatty acid chain length, extent of fatty acid unsaturation, and PC/PE ratio. The results are summarized in Table 1 and in the following figures.

Figure 4 displays the variation of the binding enthalpy, $\Delta H°$, with temperature. For 100 nm vesicles (same lipid composition as in Figure 2), $\Delta H°$ decreases linearly with temperature and becomes positive at temperatures below 9.5 °C. In contrast, a nonlinear behavior is observed for 30 nm vesicles. At low temperatures, $\Delta H°$ remains almost constant but is still distinctly exothermic. At higher temperatures, the $\Delta H°$ (30 nm) approaches the $\Delta H°$ (100 nm) values.

The temperature dependence of the binding constant for complex formation, K_0, is shown in Figure 5 for 30 and 100 nm vesicles. The open symbols represent the experimental data, the solid lines are the theoretical prediction of the K_0 vs T curve, based on the van't Hoff relation $d \ln K/dT = \Delta H°/RT^2$, and the known temperature dependence of $\Delta H°$ (taken from Figure 4). Within the accuracy of the measurement, a good agreement between experimental results and theory is obtained. It should be noted that the peptide-lipid binding constant of 100 nm vesicles is about a factor 5 stronger than that of 30 nm vesicles at the same temperature.

The influence of the fatty acyl chain length was investigated by preparing mixed POPC/PE vesicles (10:1 and

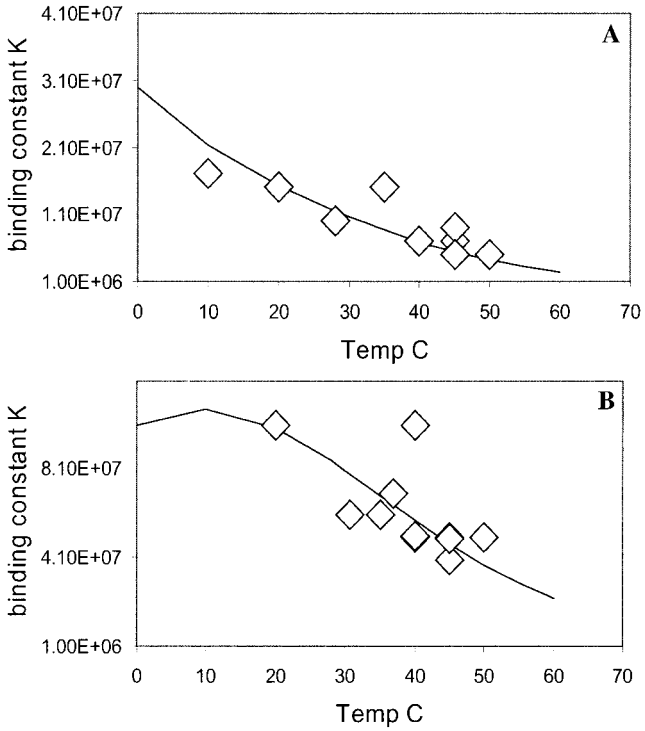

FIGURE 5: Temperature dependence of the binding constant of PE:Ro 09−0198 complex formation. (A) 30 nm vesicles composed of POPC/POPE (10:1 wt/wt) (B) 100 nm vesicles POPC/POPE (10:1 wt/wt). The solid lines are calculated with the measured temperature dependence of $\Delta H°$ (Figure 4) using the van't Hoff relation $d \ln K/dT = \Delta H°/RT^2$.

∼100:1 wt/wt) in which 1,2-diacyl-*sn*-glycero-3-phosphoethanolamines with fatty acyl chains varying in chain length between $n = 8$ and $n = 14$ carbon atoms were employed. The binding constants and reaction enthalpies were very similar for all lipid species and are detailed in Table 1. As a control experiment Ro 09-0198 (40 μM) was titrated with glycerophosphorylethanolamine (1.2 mM) since GPE is known not to bind to Ro 09-0198 (*2*). No heat of binding was observed.

Finally, we have titrated Ro 09-0198 (200 μM) with pure POPC vesicles (SUVs). A small exothermic reaction was noted. The data were evaluated in terms of a partition equilibrium, $X_b = K_p c_{eq}$, where X_b denotes the mole ratio of bound peptide-to-lipid (on the vesicle outside) and c_{eq} is the peptide equilibrium concentration (*17*). No charge effects were considered. The binding enthalpy was -0.5 to -1 kcal/mol and the partition constant $100-300$ M^{-1}.

Deuterium- and Phosphorus-31 NMR. The properties of the PE/Ro 09-0198 complex in the bilayer membrane and

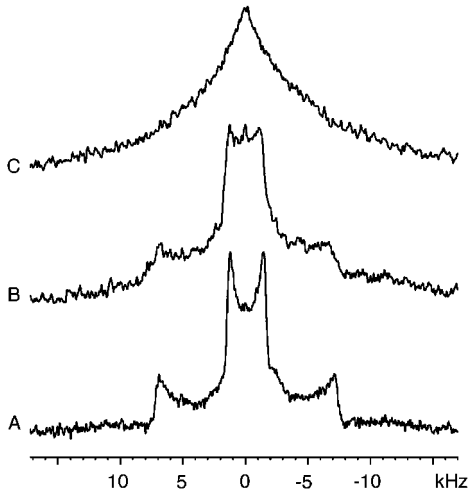

FIGURE 6: ^{2}H NMR spectra of mixed PE:PC (1:4) multilamellar dispersions in excess 2H depleted water at 30 °C. (A) Pure lipid membrane. (B) Addition of Ro 09-0198 at a PE/Ro 09-0198 ratio of 1:0.5. (C) PE/Ro 09-0198 ratio of 1:1.

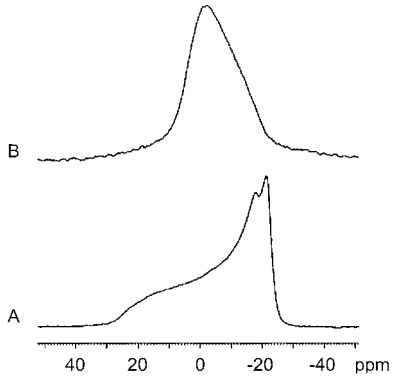

FIGURE 7: ^{31}P NMR spectra of mixed PE/PC (1:4) multilamellar dispersions in excess water at 30 °C. (A) Pure lipid membrane. (B) Addition of Ro 09-0198 at a PE/Ro 09-0198 ratio of 1:1.

its influence on the surrounding noncomplexed lipids were investigated with solid-state NMR techniques. To this purpose, a POPE molecule was synthesized which was deuterated at the *cis*-double bond of the *sn*-2 oleic acyl chain ([9′,10′-$^2H_2$]POPE). The lipid was mixed with nondeuterated POPC at a POPC/POPE ratio of ~4:1 (wt/wt), i.e., the membrane contained twice as much PE as in the calorimetric experiments. This was necessary to generate sufficient ^{2}H NMR signal intensity without increasing the sample size too much. Typically, the NMR sample was composed of 10−15 mg of deuterated PE, 40−60 mg of PC and 100 μL of deuterium depleted water. PE contents larger than 20% are quite common in bacterial membranes. Ro 09-0198 was added at a total PE/peptide ratio of 1:0.5 and 1:1. Figure 6 then shows deuterium NMR spectra of mixed PE/PC multilamellar dispersions at 30 °C. The deuterated PE in the pure lipid membrane gives rise to two quadrupole splittings (Figure 6A) with separations of 14.1 and 2.7 kHz which can be assigned to the C-9 and the C-10 deuteron of the *cis*-double bond (*11*). The spectrum is typical for a liquid-crystalline lipid bilayer (*18*) which is also confirmed by the phosphorus NMR spectrum shown in Figure 7A (*19*). Distinct changes in both the deuterium and phosphorus spectra are observed upon addition of Ro 09-0198. Figure 6, panels B and C compare ^{2}H NMR spectra at a PE/peptide ratio of 1:0.5 and 1:1, respectively. At the 1:0.5 ratio, PE is not fully complexed. The NMR spectrum shows a superposition of two compounds, namely, (i) the noncomplexed POPE with two quadrupole splittings virtually identical to those observed in the absence of Ro 09-0198 and (ii) a broad featureless spectrum. The second component can be visualized in pure form in Figure 6C which represents the ^{2}H NMR spectrum of a PE:Ro 09-0198 (1:1) complex embedded in a POPC matrix. The spectrum is no longer characteristic of an axially symmetric motion. The corresponding phosphorus-spectrum is shown in Figure 7B.

DISCUSSION

Ro 09-0198 belongs to a group of peptidic toxins (lantibiotics) characterized by the presence of the unusual thioether amino acids lanthionine and methyllanthionine (*4*). Among these, the linear peptide nisin Z was recently shown to interact specifically with the membrane-anchored cell-wall precursor lipid II (*N*-acetylglucosamine-β-1,4-*N*-acetyl-muramyl-pentapeptide-pyrophosphoryl-undecaprenol). The cationic nisin Z ($z = 3-4$) combines a high affinity for lipid II with a pore-forming potential, killing its target by permeabilizing the plasma membrane (*20*).

The interaction of the globular, electrically neutral Ro 09-0198 with phospholipids has also been analyzed on the basis of its hemolytic activity (*1*, *2*). The previous findings can be summarized as follows: (i) Ro 09-0198 binds specifically to phosphatidylethanolamines but not to other phospholipids. (ii) The correct phosphoethanolamine headgroup and at least one hydrophobic acyl chain are necessary for optimal interaction with the peptide. (iii) Ro 09-0198 recognizes PE in both model membranes and biological membranes.

The present study then confirms the 1:1 complex formation and provides quantitative insight into the thermodynamics of the binding process as summarized in Table 1 and Figures 4 and 5. Binding constants for 30 and 100 nm vesicles (POPC/POPE 10:1 wt/wt) are shown as a function of temperature in Figure 5. They fall in the range of 0.5×10^7 to 10^8 M^{-1}, and the dissociation constants are accordingly $K_D \sim 10$ to 200 nM. Hence, the free energy of complex formation, $\Delta G° = -RT \ln K_0$, varies only slightly between $\Delta G° = -10.3$ kcal/mol at 10 °C and −11.2 kcal/mol at 50 °C.

In contrast, $\Delta H°$ of 100 nm vesicles decreases distinctly from ~0 kcal/mol at 10 °C to −9.8 kcal/mol at 50 °C; the entropy term $T\Delta S$ decreases in parallel from $T\Delta S = 10.3$ kcal/mol (10 °C) to 1.4 kcal/mol (50 °C). It can be concluded that complex formation between PE and Ro 09-0198 is an entropy-driven reaction at 10 °C but is almost completely enthalpy-driven at 50 °C. Since $\Delta G°$ decreases only by 0.9 kcal/mol, the temperature dependence of the PE/Ro 09-0198 complex formation reveals an enthalpy−entropy compensation mechanism.

The structure of the Ro 09-0198/C12-lyso-PE complex in DMSO has been solved with ^{1}H NMR (*8*). The peptide has a pocket of sufficient size to accommodate the glycerophosphoethanolamine headgroup as a tight complex. Improved binding of the ligand would lower the enthalpy but tighter binding would also entail a loss of entropy of both reaction partners. Alternatively, loser binding would favor the entropic term but would also diminish the enthalpic

contribution. In both cases the changes in free energy remain small (for a more detailed discussion of enthalpy−entropy compensation, see refs *21*−*23*). Computer modeling of Ro 09-0198 with phospholipids has produced different interaction models of similar energy suggesting a peptide groove of limited flexibility (*24*). The peptide groove opens up if lyso-phosphatidylethanolamine is replaced by diacyl phosphatidylethanolamine. These studies do not give an explanation of enthalpy−entropy compensation, but they at least indicate mechanisms of tighter or looser binding.

The binding reaction must be preceded by a loss of water molecules around the peptide and the lipid headgroup. This is indeed reflected in the temperature dependence of the reaction enthalpy. For 100 nm vesicles, $\Delta H°$ follows the relationship $\Delta H°$ (kcal/mol) $= -0.2415$ T (°C) $+ 2.3$. The large negative heat capacity of the complexation reaction, $\Delta C_p = -245$ cal/mol, is characteristic for the transfer of nonpolar substances from water into a hydrophobic environment and is known as "hydrophobic effect" (cf. ref *25*). A simplified picture can be given as follows: since both PE and Ro 09-0198 are hydrated, the molar heat capacities are essentially determined by the two large hydration shells. The binding reaction then releases some of this hydration water resulting in a negative ΔC_p value for complex formation.

The titration experiments reported above were performed with vesicles composed of POPC/PE at a 10:1 molar ratio. The effective PE concentration, C_L^0, used in the simulations was however always different from the weighing-in concentrations by a "lipid availability factor" $f_L < 1$. For 30 nm vesicles the lipid availability factor was $f_L = 0.70 \pm 0.05$ ($n = 8$ titrations), for 100 nm vesicles $f_L = 0.56 \pm 0.07$ ($n = 13$). A purely statistical distribution of PE between inner and outer monolayer would predict f_L (30 nm) ∼0.6 and f_L (100 nm) $= 0.5$. It can be concluded that the addition of Ro 09-0198 has little effect on the transbilayer movement of PE as long as the PE concentration is low (≤ 10%). Several reasons could explain the small discrepancies between theory and experiment: (i) incorrect lipid concentrations, (ii) a small preference of PE for the lipid outer surface in curved membranes, and/or (iii) slow and limited PE translocation from the inner to the outer half-layer induced by peptide binding. When the analogous titrations were performed with vesicles containing more than 20 mol % PE, the lipid availability factor was $f_L = 1$ without a change in the other thermodynamic parameters (cf. Table 1). At higher PE concentrations a fast flip-flop of PE sets in, probably induced by the perturbation of the bilayer by the lipid-peptide complex as visualized with ^{2}H- and ^{31}P-NMR. Indeed, Ro 09-0198 was shown to have hemolytic and membrane permeabilizing properties suggesting a temporary disruption of the bilayer membrane.

Our titration experiments with pure POPC bilayers demonstrate that Ro 09-0198 readily partitions into membranes not containing PE. However, the measured reaction enthalpies are small and are only about −1 kcal/mol (SUVs). The corresponding binding constants are in the range of 100−300 M^{-1} and the free energies of binding are $\Delta G° \sim -5.0$ to −6.0 kcal/mol (calculated according to $\Delta G° = -RT \ln 55.5\ K_p$ (*17*)). These thermodynamic parameters are of the same order of magnitude as found for the nonspecific partitioning of the related lantibiotic nisin Z into POPC bilayers ($K_p \sim 540\ M^{-1}$, $\Delta H° = -3.4$ kcal/mol and $\Delta G° = -6.1$ kcal/mol (*26*)) indicating that both peptides experience similar hydrophobic interactions with the lipid matrix.

The NMR measurements provide insight into two structural aspects: deuterium NMR monitors the motion of the labeled PE molecules whereas phosphorus NMR reflects the structural organization of the whole lipid matrix (POPC/POPE 4:1 molar ratio). In the absence of peptide, the phosphorus spectra correspond to the classical bilayer spectra. A distinction can be made between POPC and POPE since the latter has a somewhat smaller residual chemical shielding anisotropy. A dramatic reorganization of the membrane is observed after complex formation (POPE/Ro 09-0198 1:1 complex). The phosphorus spectrum is now dominated by a broad, almost isotropic peak with only a small fraction of lipid remaining in a bilayer structure. Since POPE accounts for only 20% of the total phospholipid it is obvious that the perturbation of the bilayer extends beyond the lipid-peptide complex proper and also affects a large fraction of the remaining POPC lipids.

The deuterium NMR spectrum of POPE in the pure lipid bilayer is characterized by two quadrupole splittings which can be assigned to the two deuterons of the *cis*-double bond. The T_1 relaxation time is 15 ms (at 25 °C), corresponding to a molecular correlation time of 1.6×10^{-10} s. Addition of Ro 09-0198 at a POPE/peptide 1:1 ratio eliminates the individual quadrupole splittings and produces a broad featureless resonance as the envelope of the two splittings. The change in the T_1 relaxation time is much less dramatic, decreasing by about 25% to 11.8 ms ($\tau_c \sim 2.0 \times 10^{-10}$). The line shape can be explained by a slow isotropic tumbling motion of the complex in the remaining bilayer structure. The small change in T_1 provides evidence that the fast segmental motions of the *cis*-double bond are not much affected by complex formation. Taken together, the two types of spectra provide unambiguous evidence for a distinct perturbation of the bilayer structure by the PE/peptide complex explaining the hemolytic activity of Ro 09-0198. In light of the phosphorus NMR experiments, pore formation via the specific aggregation of peptide molecules appears to be rather unlikely.

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