Membrane Perturbation by the Lipopeptide Surfactin and Detergents as Studied by Deuterium NMR

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Surfactin is a lipopeptide produced by bacillus subtilis, which has been shown to permeabilize cell and model membranes by an unspecific mechanism often referred to as detergent-like. We have compared the structural effects of surfactin on lipid membranes with those of two nonionic detergents, $C_{12}EO_6$ and $C_{12}EO_8$, by means of solid-state NMR of selectively deuterated lipids. The detergents exhibit the expected behavior of increasing the lateral pressure in the headgroup region and disordering the acyl chains. In contrast, the strong activity of surfactin to destabilize membranes is not reflected in an extreme disordering of the fatty acyl chains. However, surfactin tilts the acyl chains of the lipid and leads to a reorientation of the lipid headgroup toward the membrane interior. These effects provide evidence for a rather deep insertion of the peptide moiety into the hydrophobic−hydrophilic interface of the membrane. The results are discussed in terms of the molecular parameters governing the activity of a molecule to destabilize lipid membranes and the activity of antibiotic peptides to induce unspecific leakage of membranes.

Introduction

Antimicrobial peptides have become a focus of interest as an alternative to classical antibiotics that suffer from the appearance of resistant bacteria. Most authors share the opinion that antibiotic peptides kill a target cell by permeabilizing its membrane. There is also some consensus that the peptide molecules accumulate in the outer interface of the membrane until membrane leakage is triggered at a characteristic concentration. (For reviews, see McElhaney and Prenner[1] and Lohner and Epand.[2])

Different models are discussed to explain the mechanism of membrane permeabilization. The large structural variability of the membrane-active peptides argues in favor of a variety of different mechanisms, also including rather unspecific modes of action. There is evidence for the formation of channel-like oligomers by amphipathic peptides such as magainin or PGLa.[3,4] However, these and other peptides may also act rather nonspecifically in a detergent-like manner (e.g., refs 5−9).

Surfactin is a mixture of closely related lipopeptides produced by bacillus subtilis.[10] It consists of a heptapeptide unit closed to a lactone ring by a β-hydroxy fatty acid containing about 14−15 carbons. The most abundant sequence is Glu-Leu-D-Leu-Val-Asp-D-Leu-Leu. Surfactin has hemolytic,[11] antiviral,[11,12] antibacterial,[13,14] and antitumor[15] properties.

Detergents induce a positive spontaneous curvature in membranes, leading to a disordering of the acyl chains,[16] a reduction in membrane thickness and lateral packing density, and a loss of mechanical membrane stability.[17] If unable to flip to the inner monolayer, they create tension in the bilayer described by the bilayer couple concept.[18] This effect can provide sufficient energy for transient membrane rupture.[19] Because detergents typically prefer a large positive interfacial curvature, they can stabilize leaks in the membrane by forming a rim around the edge of the leak, thus shielding the hydrophobic core from water (e.g., ref 20).

Peptides can induce similar membrane effects (e.g., a spontaneous curvature in membranes[2,21,22]). Amphipathic helices in an in-plane orientation can substantially reduce membrane thickness.[23] The stress due to asymmetric insertion into the outer monolayer is supposed to promote the transient movement of a helical peptide from an in-plane to a trans-membrane orientation, which is a prerequisite for specific pore formation.[24] The term "detergent-like" action of peptides is used for unspecific mechanisms of membrane permeabilization. Ladokhin and White[9] defined detergent-like behavior by the absence of a size threshold for the permeating solutes, which is in contrast to permeation through pores that have a well-defined pore diameter. Lohner and Epand[2] distinguished "bilayer defects" from "pores" by the presence or absence of increased flip-flop of the lipid. Despite qualitative similarities, peptides can be far more efficient in destabilizing membranes than strong synthetic detergents.[8] This raises the main question of our study: whether and how the structural perturbations of the lipid membrane induced by peptides differ from those of detergents.

Another open issue regarding the behavior of surfactin is its localization in the membrane. Bonmatin et al.[25] proposed a horse-saddle structure of the peptide ring in solution, with the two charged residues pointing away from the hydrophobic tail. Recent molecular dynamics studies argued that such a structure would also be formed at a hexane−water interface, thus allowing most of the apolar groups to reside in hexane whereas the Glu and Asp are in water.[26] Such behavior would be energetically quite favorable, taking into account the considerable free energy gain of up to −23 kJ/mol of burying 4 Leu and 1 Val in a hydrophobic phase according to the MPEx program.[27] This program is based on the Wimley and White[28] scale, which not only considers the side-chain interactions but also takes into account the polar nature of the backbone. The free-energy gain calculated above would be an upper limit because the Glu-1

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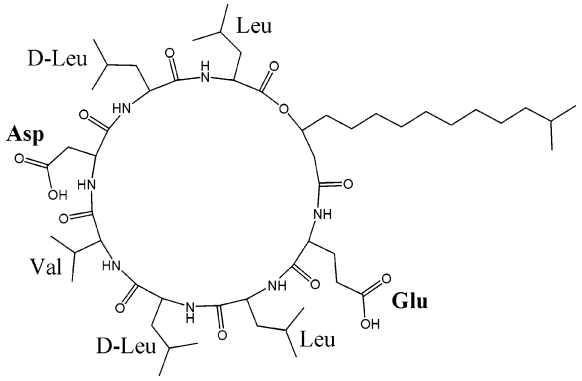

Figure 1. Chemical structure of the major component of surfactin.

and Asp-4 residues must be localized in the aqueous phase and prevent a complete immersion of the cyclic peptide in the hydrophobic membrane core. Nevertheless, the Glu and Asp residues may "snorkel" into the water whereas most of the hydrophobic amino acids penetrate into the hydrophobic core. This is, however, not in line with an X-ray scattering and DSC study[29] suggesting a peripheral localization of the peptide.

The present study investigates structural effects exerted by surfactin and detergents on the headgroup and chain regions of the membrane by means of solid-state NMR. The size of the ^{2}H quadrupole splitting of selectively deuterated lipids depends on both the molecular conformation and the extent of molecular fluctuations of the labeled segment. Neutron diffraction and ^{2}H NMR studies have led to a detailed picture of the phosphocholine headgroup structure in bilayer membranes. The $^-$P$-$N$^+$ dipole is close to an in-plane orientation and makes an angle of $\sim$30° with the bilayer suface.[30−32] Moreover, the phosphocholine group is sensitive to the electric surface charge on the membrane. Negative surface charges move the $^+$N end of the $^-$P$-$N$^+$ dipole closer to the membrane surface, which is reflected by an increase (decrease) in the quadrupolar splitting of lipid deuterated selectively at the α segment (β segment) of the choline group.[33,34] Cationic additives[33,34] and, to a much lesser extent, nonionic detergents[35−37] have the opposite effect.

The effect of surfactin and detergents on the acyl chain structure was studied using POPC deuterated at the cis double bond of the oleic acyl chain (carbon atoms C-9, C-10), which produces membrane spectra with two quite different quadrupole splittings (Figure 3), $|\nu_9| = 13.9$ kHz and $|\nu_{10}| = 1.8$ kHz in pure POPC. Because the cis double bond has no internal flexibility, the two C$-$D bonds undergo the same fluctuations. The two different quadrupole splittings must therefore arise from different orientations of the two C$-$D bond vectors with respect to the bilayer normal.[38] Indeed, the C$=$C double bond is slightly tilted with respect to the normal to the bilayer surface (which is the axis of motional averaging) which brings the $C_{10}-$D vector close to the magic angle (small splitting) and the C_9-D bond vector farther away from it (large splitting). Superimposed on these geometric effects are motional fluctuations that reduce both splittings to an equal extent.

Materials and Methods

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was deuterated selectively at the α- ($-NCH_2CD_2OP-$) or β-($-NCD_2CH_2OP-$) methylene segment of the choline headgroup moiety or at the carbon atoms C-9,10 of the oleic chain (at the cis double bond), as described previously.[38,39] The purity was checked by thin-layer chromatography and in terms of the

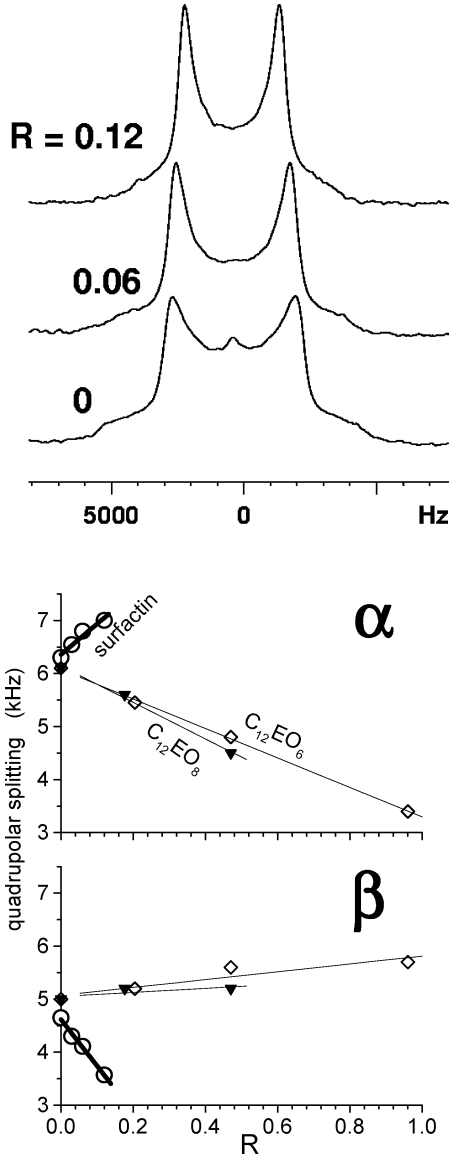

Figure 2. Effect of surfactin on the POPC lipid headgroup at 25 °C. (Upper panel) ^{2}H spectra of POPC deuterated at the β-headgroup segment at different surfactin-to-lipid mole ratios, *R*. (α and β) Quadrupolar splitting of POPC labeled in the α and β positions, respectively, as a function of the mole ratio *R* of either surfactin, $C_{12}EO_6$, or $C_{12}EO_8$ to the lipid. The slopes of the fit lines are listed in Table 1.

quadrupolar splitting of the pure lipid. Surfactin was purchased from Sigma (St. Louis, MO). The detergents hexaethyleneglycoldodecyyl ether ($C_{12}EO_6$) and octaethyleneglycoldodecyl ether ($C_{12}EO_8$), tris buffer, EDTA, and NaCl were from Fluka (Buchs, Switzerland). All buffers were made in deuterium depleted water. A buffer of 110 mM tris and 1 mM EDTA (pH 8.5) was used for samples containing surfactin. The detergent samples were in 10 mM tris, 100 mM NaCl at pH 7.4.

Each sample contained approximately 10 mg of lipid. The lipid was dissolved in dichloromethane, and the appropriate amount of the stock solution was filled into a cylindrical NMR test tube (8 mm). The solvent was removed by a gentle stream of nitrogen. Finally, the film was dried overnight at high vacuum. After the addition of 100 μL of buffer to the dry lipid, the suspension was subjected to five freeze$-$thaw cycles. Surfactin was either mixed with the lipid in organic solvent or dissolved in the buffer and added to the dry lipid; both procedures are assumed to give rise to a homogeneous distribution of surfactin over all layers of the multilamellar vesicles

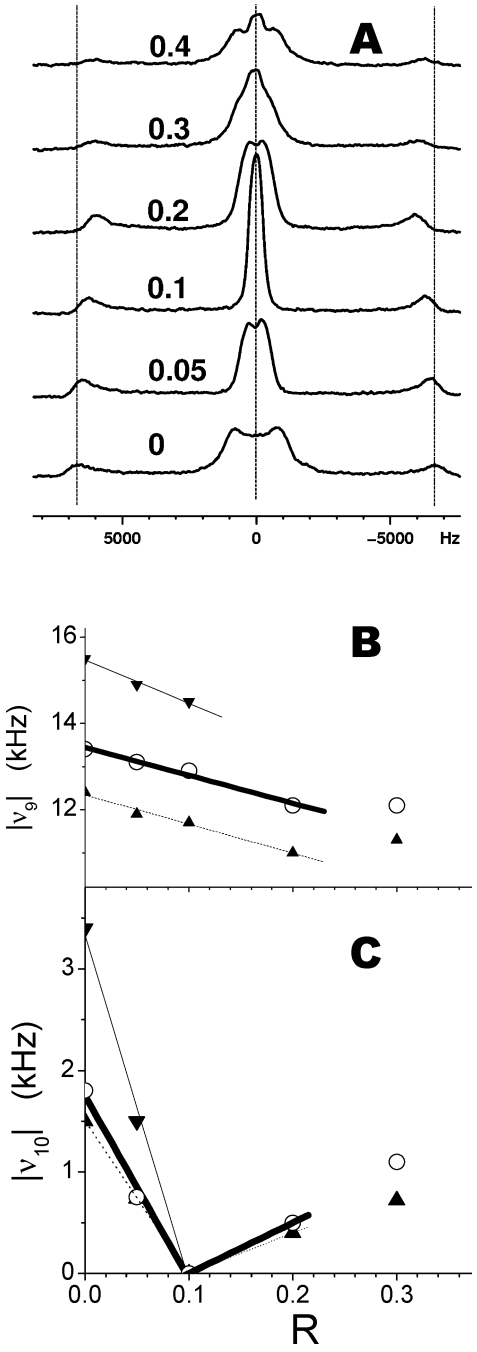

Figure 3. Effect of surfactin on the lipid acyl chains. (A) ^{2}H NMR spectra of [9,10-D_2]-POPC vesicles at 25 °C containing various amounts of surfactin as specified in terms of the surfactin-to-lipid mole ratio, *R*. (B, C) Quadrupolar splittings of deuterium at the C_9 (B) and C_{10} (C) positions measured at 45 °C (▲), 25 °C (○), and 5 °C (▼) shown as a function of *R*. Membrane lysis starts at *R* between 0.2 and 0.3 at 25 °C.

and led, indeed, to the same spectra. The high mole ratio partition coefficients and low critical micelle concentrations of surfactin (K = 22 mM^{-1}, cmc = 7.5 μM),[8] $C_{12}EO_8$ (K = 6 mM^{-1}, cmc = 90 μM),[40] and $C_{12}EO_6$ (K = 20 mM^{-1}, cmc = 65 μM)[40] imply that these additives are virtually completely (>99.8%) incorporated into the lipid membranes and aqueous monomers are negligible.

^{2}H NMR spectra were recorded on a Bruker DRX 400 NMR spectrometer operating at 61.43 MHz. We employed a quadrupole echo technique with 90° pulses of 6-μs width, an echo time of 32 μs, and a repetition time of 240 ms.[41] Typically, some 10 000 scans were recorded for each spectrum.

The absolute value of the quadrupole splitting, $|\nu|$, is given by the separation of the most intense peaks in the spectrum. For a random distribution of bilayer domains, $|\nu|$ is given by

$$\nu = \frac{3}{4}\left(\frac{e^2qQ}{h}\right)\cdot S_{CD} \quad (1)$$

where the quadrupole coupling constant, (e^2qQ/h), is 170 kHz for an aliphatic C−D bond. S_{CD} is the deuterium order parameter defined as

$$S_{CD} = 0.5\cdot(3\langle\cos^2\Theta\rangle - 1) \quad (2)$$

Θ is the instantaneous angle between the C−D bond vector and the normal to the bilayer surface. The average orientation as reflected by $\langle\cos^2\Theta\rangle$ depends on both the geometry of the molecule (orientation of the C−D bond with respect to the molecular axis of rotation) and the extent of fluctuations of the molecular axis around the bilayer normal.

Results

Headgroup Structure. The top panel of Figure 2 shows spectra of POPC deuterated at the β segment of the choline group at different surfactin-to-lipid molar ratios, *R*. Note that the total ratio, *R*, agrees with the local ratio within the membrane because monomers are negligible. (See Materials and Methods.) The spectra are typical for a powder-type distribution of lipid bilayers. The quadrupole splitting $|\nu_\beta|$, defined as the separation of the most intense peaks in the spectrum, amounts to ~5.0 kHz for POPC at room temperature.

The figure demonstrates that the quadrupole splitting of the β-CD_2 segment decreases with increasing surfactin concentration. The analogous experiment performed with POPC deuterated at the α segment leads to the opposite result; the quadrupole splitting increases with increasing surfactin concentration (spectra not shown). The quantitative analysis of the α- and β-quadrupole splittings is given in the bottom panel of Figure 2. The bilayer membrane is stable up to about 20% surfactin in the membrane. Above a "saturating" detergent-to-lipid mole ratio in the membrane, $R_b^{sat} = 0.22$, micellar structures are found (see below).

For $C_{12}EO_6$ or $C_{12}EO_8$, much larger amounts can be accommodated in the POPC membrane. The effects of these two surfactants and octyl-β-glucopyranoside (OG, data from Wenk et al., ref 36) are clearly smaller than those of surfactin at the same *R* value (see Table 1). In addition, the variations of the quadrupole splittings with detergent concentration are in the opposite direction to those seen with surfactin. The α splitting decreases and that of the β splitting increases (Figure 2, bottom panel).

Figure 2 further demonstrates that the change in quadrupole splitting is virtually linear with the surfactant-to-lipid ratio. Let us define the slope of a quadrupole splitting as a function of the mole ratio *R* as *m*:

$$m = \frac{d|\nu|}{dR} \quad (3)$$

Hence, m_α and m_β can be considered to be quantitative measures of the efficacy of a given surfactant to influence the headgroup conformation. Table 1 summarizes the *m* values derived for the various combinations of segments and surfactants investigated.

Chain Structure. The central part of the bilayer was probed with POPC deuterated at the cis double bond of the sn-2 oleic acyl chain. Figure 3A shows ^{2}H NMR spectra of [9′, 10′-D_2]

TABLE 1: Variation of the Quadrupolar Splittings of POPC Deuterated at Different Positions in the Head Group (α or *β*) and the Oleic Chain (C_9 and C_{10}) as a Function of the Mole Ratio of Additive Per Lipid, *R*[a]

	OG	$C_{12}EO_6$	$C_{12}EO_8$	surfactin		
T/°C	25	25	25	45	25	5
			Membrane Destabilization			
K·cmc[40]	2.3	1.3	0.54		0.2	
R_b^{sat}	1.4[40,42]	1.2 [43]	0.57 [44,45]	~0.4[b]	0.22[b]	~0.1[b]
			Structure Of Lipid Headgroups			
$\lvert\nu_\alpha\rvert$ at R_b^{sat} (kHz)	3[36]	3	4		7	
m_α (kHz)	−2.1[36]	−2.8	−3.4		+5.8	
m_β (kHz)	+0.9[36]	+0.7	+0.4		−8.8	
m_β/m_α	−0.4	−0.25	−0.1		−1.5	
			Structure of the Acyl Chains			
$\lvert\nu_9\rvert$ at R_b^{sat} (kHz)	11.5[36]	9.2	9.9		12	
m_9 (kHz)	−1.1[36]	−3.4	−6.0	−6.7	−6.5	−10
m_{10} (kHz)				−15 (R ≤ 0.1)	−18 ($R \le 0.1$)	−34
				+4 (R ≥ 0.1)	+5 ($R \ge 0.1$)	

[a] Linear fits are shown in Figures 2−4. The product of the partition coefficient and the critical micelle concentration can be considered to be an index for membrane destabilization.[40] R_b^{sat} denotes the surfactant-to-lipid mole ratio at the onset of solubilization. [b] The values for surfactin are consistent with the appearance of an isotropic signal in the ^{2}H NMR spectra and agree with results of independent ITC and fluorescence measurements (Heerklotz and Seelig, to be submitted for publication).

POPC bilayers at various surfactin-to-lipid ratios. The spectra show two quadrupole splittings that can be assigned to the C-9 (large splitting) and the C-10 (small splitting) deuterons.[38] As surfactin is added to the POPC membrane, the C-9 splitting decreases smoothly with a slope of −4.7 kHz (at 25 °C) (cf. Figure 3C).

In contrast, the C_{10} segment exhibits unusual behavior. Initially, $\lvert\nu_{10}\rvert$ decreases with increasing surfactin content ($m_{10} \approx -18$ kHz) until the spectrum collapses to a single line ($\lvert\nu_{10}\rvert \approx 0$) at $R \approx 0.1$. If more surfactin is added, then the quadrupole splitting reemerges (cf. spectra at $R = 0.1$ and 0.2). A plot of $\lvert\nu_{10}\rvert$ versus R thus exhibits a discontinuity at $R \approx 0.1$. However, it should be noted that the quadrupole splitting is a signed quantity and that the sign cannot be determined in a conventional ^{2}H NMR experiment. A continuous change of ν_{10} is observed if the quadrupole splitting for $R > 0.1$ has the opposite sign to that for $R < 0.1$. The stronger decrease of $\lvert\Delta\nu_{10}\rvert$ relative to that of $\lvert\Delta\nu_9\rvert$ with increasing surfactin content in the membrane and, in particular, the collapse and reemergence of the C_{10} splitting must therefore be interpreted as follows. The addition of surfactin causes a change in the tilt angle of the cis double bond such that the angle between the C_{10}−D bond vector and the bilayer normal (i.e., the axis of motional averaging) comes close to the magic angle. At higher R, the tilt angle increases further, now moving the C_{10}−D bond gradually away from the magic angle so that the splitting reemerges and increases with R. Unspecific disordering of the membrane by surfactin contributes to the decrease in $\lvert\nu_{10}\rvert$ at $R < 0.1$ but counteracts the reemergence of the splitting at $R > 0.1$. This accounts for the change in the slope, m_{10}, at $R = 0.1$.

As mentioned above, membrane disintegration starts at $R_b^{sat} = 0.22$. This limit has been derived on the basis of isothermal titration calorimetry studies (ref 8 and Heerklotz and Seelig, to be submitted for publication). It is confirmed here by ^{2}H NMR. Inspection of the ^{2}H NMR spectra in Figure 3 for $R = 0.3$ and 0.4 shows a signal with no measurable quadrupole splitting superimposed on the small C-10 quadrople splitting. This signal is indicative of a fraction of lipid that reorients much faster with respect to the external field, most likely arising from lipids in nonspherical micelles.

Analogous experiments using [9,10-$^2H_2$] POPC were performed with $C_{12}EO_6$ and $C_{12}EO_8$, and the data are summarized in Figure 4. Again, both quadrupole splittings are reduced upon

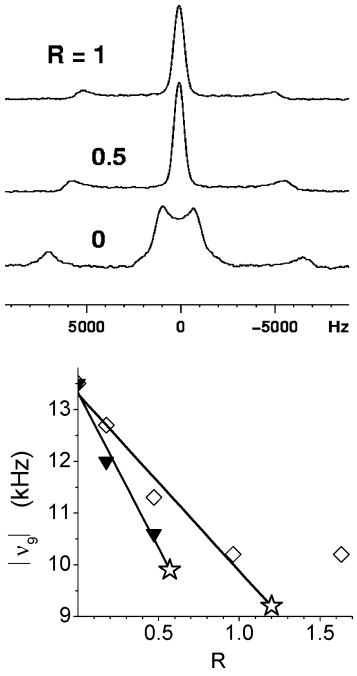

Figure 4. Effect of detergents on the lipid acyl chains at 25 °C. (Upper panel) Spectra of POPC labeled in the C_9 and C_{10} positions of the oleic acyl chain in the absence and presence of $C_{12}EO_6$. (Mole ratios of detergent per lipid R are given in the plot.) (Bottom panel) Quadrupolar splitting of the C_9 position as a function of R for $C_{12}EO_6$ (◇) and $C_{12}EO_8$ (▼). Stars (☆) illustrate the extrapolated value of $\lvert\nu_9\rvert$ at the onset of solubilization, $R = R_b^{sat}$. The lines represent linear regressions within the exclusively lamellar range ($R < R_b^{sat}$); the parameters are given in Table 1.

the addition of surfactant, and $C_{12}EO_8$ (the surfactant with the larger headgroup) is more effective than $C_{12}EO_6$ as measured by the slope of the straight lines in Figure 4 (cf. Table 1). The C_{10} quadrupole splitting collapses to a singlet resonance at $R \approx 0.5$ for $C_{12}EO_8$ ($R \approx 1$ for $C_{12}EO_6$) but, in contrast to surfactin, does not reappear at higher detergent concentrations. For POPC bilayers containing $C_{12}EO_6$, the saturation limit is $R_b^{sat} = 1.2$. At higher concentrations of detergent, the lamellar phase coexists with a micellar phase. In Figure 4, the largest detergent-to-lipid ratio was $R = 1.6$. The corresponding quadrupole splitting is, however, close to that measured slightly below saturation at $R = 1$, consistent with a bilayer saturation

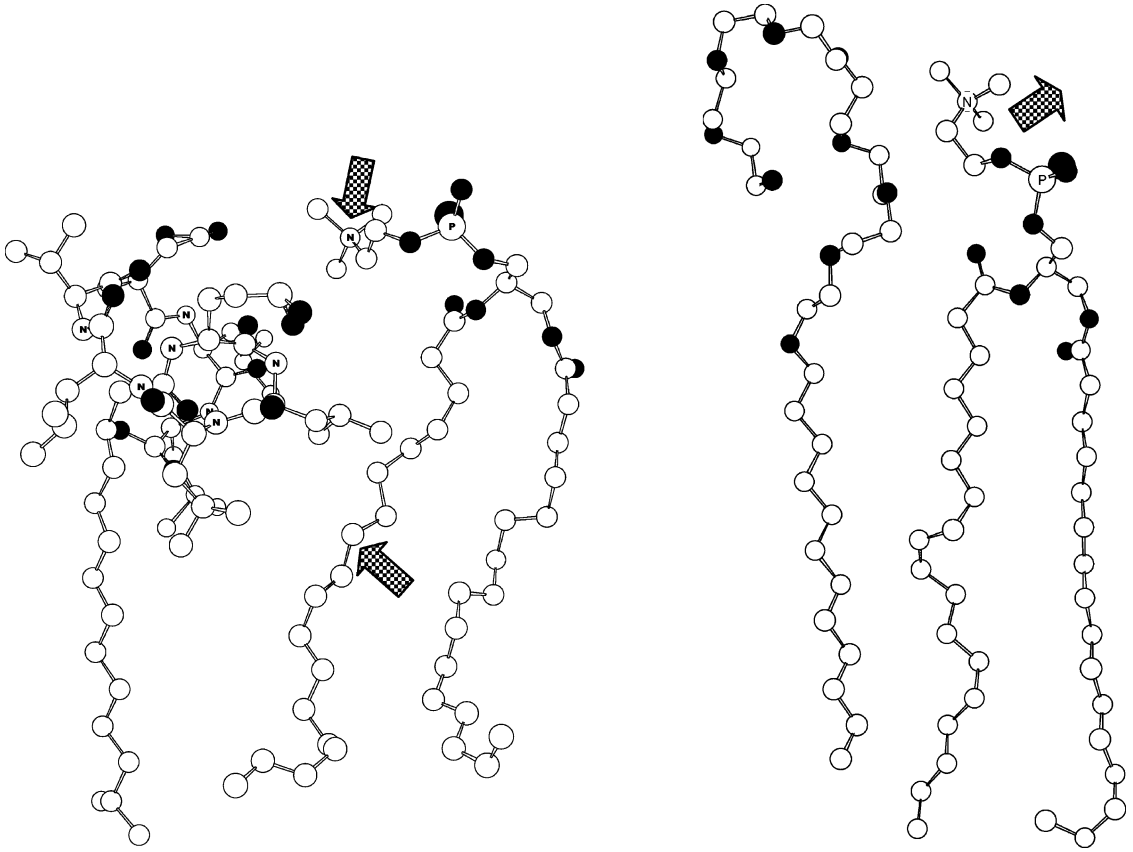

Figure 5. Schematic representation of the molecular dimensions and hypothetical localizations of surfactin (left) and $C_{12}EO_8$ (right) relative to the lipids in a membrane. Carbon atoms are shown in white, and oxygen, in black. Nitrogen, atoms are labeled with N, and phosphorus, with P. Hydrogens are omitted. The lipid carries a positive charge at the N and a negative charge at the P−O; the peptide has negative charges at Asp and Glu. Arrows indicate the structural changes of the lipid induced by the additives (see text).

level of $R = 1.2$. A linear fit of the splittings was thus restricted to the lamellar range. The results are included in Table 1.

Discussion

Disordering and Tilt of the Acyl Chains. Most detergents are characterized by a large polar headgroup and a hydrophobic tail with a distinctly smaller cross-sectional area. For example, the headgroup of $C_{12}EO_8$ has a cross-sectional area of about 54 Å^2 whereas its single fluid chain requires only ~27 Å^2 of lateral space.[46] The detergent thus adopts the effective shape of an inverted cone[47] (cf. Figure 5), leading to a positive spontaneous curvature of its aggregates and a destabilization of lipid membranes upon insertion of the detergent. The activity of a surfactant to destabilize membranes can be quantified in terms of the product of the partition coefficient and the critical micelle concentration, K·cmc. Another parameter representing the strength of a surfactant is the surfactant-to-lipid mole ratio that is required for the onset of membrane solubilization, R_b^{sat}. Both quantities are shown in Table 1 for the compounds studied here and for octyl glucoside investigated by Wenk et al.[36] The data imply that the membrane-destabilizing activity of the detergents increases from left to right in the order OG < $C_{12}EO_6$ < $C_{12}EO_8$ < surfactin. Furthermore, the activity of surfactin increases with decreasing temperature.

Next, the structural changes induced by the different molecules can be compared with the thermodynamic parameters of destabilization. The disordering of the acyl chains as indicated by m_9 increases with increasing detergent strength (from left to right in Table 1), but the high dissolving power of surfactin is not reflected in a strong further increase in m_9. Interestingly, membranes at the onset of solubilization by the detergents $C_{12}EO_n$, $n = 6$, 8, appear to show a characteristic degree of order. This is suggested by the fact that the two $C_{12}EO_n$ detergents solubilize membranes at a common splitting of ($|\nu_9|$ at R_b^{sat}) ≈ 9.5 kHz (Table 1, stars in Figure 4). OG has a very weak effect on ν_9 and solubilizes membranes before the value of 9.5 kHz is reached. Surfactin induces the onset of solubilization at a substantially higher value of $|\nu_9|$ (i.e., apparently at a higher degree of acyl chain order). This finding indicates that the mechanism of membrane destabilization by surfactin differs from that of the detergents and that ν_9 is a measure of detergent-induced membrane destabilization for simple detergents but does not reveal the destabilizing effect of surfactin.

Another qualitative difference between detergents and surfactin is the fact that only surfactin promotes a tilt of the acyl chains. This is evident from the fact that an increasing concentration of surfactin first produce a collapse of the quadrupolar splitting ν_{10}, which is then followed by a reappearance of the splitting. This behavior is not observed for detergents.

The temperature dependence of the quadrupolar splittings shown in Figure 3 represents the typical behavior: (i) Membranes are more ordered at lower temperature. (ii) Better-ordered membranes are more sensitive to a disordering agent. (iii) The onset of solubilization is shifted to higher R_b^{sat} upon increasing temperature (cf. Table 1). These phenomena can be explained by the fact that increasing temperature renders the spontaneous curvature of most lipid and surfactant molecules less positive (or more negative) because the hydrophobic tails are disordered and, possibly, headgroups are gradually dehydrated. Therefore, increasing temperature reduces curvature strains and opposes the formation of positively curved, micellar systems.

Structural Changes of the Lipid Headgroups. The addition of $C_{12}EO_6$ and $C_{12}EO_8$ detergents increases the lateral pressure in the headgroup region of the bilayer membrane and leads to a slight reorientation of the lipid headgroup away from the surface toward the membrane normal, reducing the steric hindrance between the headgroups compared to an in-plane orientation. This change in headgroup orientation can be detected as a decrease in the α splitting ($m_\alpha < 0$) and an increase in the β splitting ($m_\beta > 0$) of a headgroup-deuterated lipid.[36] This has been found for all of the nonionic detergents included in Table 1. The absolute values of $|m_\alpha|$ increase continuously with increasing strength of the detergent (see above), but at the same time, $|m_\beta|$ decreases. This leads to a continuous change of the ratio m_β/m_α from -0.4 to -0.1, which is in contrast to the effect of cationic additives, which are similar to detergents in decreasing $|\nu_\alpha|$ and increasing $|\nu_\beta|$ but exhibit much larger values of $|m_\alpha|$ and $|m_\beta|$ and a constant ratio of $m_\beta/m_\alpha \approx -0.5$.[48]

The effect of surfactin on the lipid headgroup orientation is counterdirectional to that observed for the nonionic detergents: $|\nu_\alpha|$ increases and $|\nu_\beta|$ decreases. This finding can be explained on the basis of electrostatic attraction between the two negative charges of surfactin and the dipolar choline group of the lipid that pulls the $^+$N terminus closer toward the membrane surface.

Let us compare the surfactin results with those obtained with two other negatively charged molecules, namely, a lipid (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol = POPG) and a peptide (cyclosporin A sulfate = CyA-SO_3^-). When POPG is mixed with a deuterated POPC bilayer, the α-CD_2 splitting increases with a slope of $m_\alpha = 12$ kHz, and the β-CD_2 splitting decreases with $m_\beta = -12.5$ kHz. The ratio m_β/m_α is -1.05.[49] The corresponding results for CyA-SO_3^- are $m_\alpha = 15.6$ kHz, $m_\beta = -17.7$ kHz, and $m_\beta/m_\alpha = -1.13$.[50] Moreover, if POPG is replaced by the doubly charged cardiolipin, then m_α and m_β also increase by a factor of 2 ($m_\alpha = 30$ kHz, $m_\beta = -25$ kHz).[49] In light of these earlier measurements, the doubly charged surfactin is remarkably inefficient in changing the headgroup structure with $m_\alpha = +5.8$ kHz and $m_\beta = -8.8$ kHz. The change in the quadrupole splittings is in the same direction but to a lesser extent than observed for other negatively charged molecules. For most interactions with negatively charged molecules, the changes in the quadrupole splittings of the α and β segments differ only in sign, leading to $m_\alpha = -m_\beta$ (or $m_\beta/m_\alpha \approx -1$) for all cases investigated. For surfactin, however, this ratio is larger with $m_\beta/m_\alpha \approx -1.5$. This can be understood if the intercalation of surfactin not only induces a conformational change but also increases the wobbling motion of the $^-$P$-$N$^+$ dipole. Whereas the conformational change leads to a counterdirectional change of the two quadrupole splittings (ν_α increases, ν_β decreases), any additional wobbling motion will reduce the two quadrupole splittings by the same factor.

These results together with those obtained from the fatty acyl chains suggest a rather deep insertion of surfactin into the membrane, as schematically illustrated by Figure 5 (left), whereas the detergent headgroup must be localized closer to the lipid$-$water interface (cf. Figure 5, right). One should, however, bear in mind that the membrane is highly dynamic and that the position of the molecules varies over a certain range. This model is also consistent with the experimental finding of Grau et al.[29] that surfactin increases the *d* spacing of fluid multilamellar DMPC vesicles by 4 Å. This effect is most likely due to the swelling of the interlamellar water layer caused by the electrostatic repulsion of adjacent layers by embedding surfactin. The general structure of the peptide shown in the figure corresponds approximately to the horse-saddle conformation established by NMR measurements in solution[25] with the two charged residues, Asp-4 and Glu-1, pointing away from the hydrophobic tail into the water and attracting the positive charge of the choline group.

Membrane-Destabilizing Activity. Both the structural data collected here and thermodynamic parameters published recently[8] imply that surfactin is a very potent membrane-perturbing agent. Three factors contribute to the membrane-disrupting properties, namely, (i) the size of the peptide ring, (ii) its anchoring force in the membrane, and (iii) its positioning with respect to the plane of the membrane.

According to the spontaneous curvature model, the most potent detergent in a family of compounds sharing the same hydrophobic part is that with the largest headgroup. The peptide ring of surfactin is, in fact, very large and can account for a very strong membrane perturbation.

However, the heptapeptide moiety is not truly polar but predominantly hydrophobic. Polar headgroups of similar size would probably be expelled from the lipid headgroup region to a more peripheral localization, thus reducing or eliminating membrane perturbation. Upon such an expulsion, most of the alkyl chain would stay in the hydrophobic core, and only a few methylene groups adjacent to the headgroup would be exposed to a polar environment. In contrast, the anchoring force of surfactin is much larger and accounts for the much larger membrane destabilization. There are one Val and four Leu residues that anchor the peptide ring tightly to the hydrophobic core of the membrane, and this effect is enhanced by the hydrophobic tail. Consequently, surfactin can induce a much larger membrane perturbation than a detergent with a truly polar headgroup of similar size.

The fact that the peptide moiety of surfactin is itself amphiphilic with a preponderance of hydrophobic residues also accounts for the deep insertion of this bulky part into the interfacial and upper hydrocarbon regions of the membrane. The outer region of the hydrophobic core exhibits the highest order.[51−53] Moderate perturbations of the chain packing such as branching or cis double bonds have only a minor effect if they occur closer to the center of the membrane but give rise to considerable perturbations if localized close to the interface. The localization in the most sensitive region of the bilayer can be considered to be another reason for the high destabilizing activity of surfactin.

In addition to the effects studied here with a homogeneous distribution of surfactin within the membrane, the charge and size of the peptide suggest that surfactin added to the medium inserts asymmetrically into the outer monolayer of vesicles or cell membranes and undergoes no fast flip to the inner monolayer. This would further destabilize the bilayer by an imbalance between the packing in the two membrane leaflets.

Concluding Remarks

Surfactin, much like synthetic detergents, induces imbalances in the lateral pressure between the headgroup and chain region (monolayer curvature strain) and between the outer and inner lipid layers of the membrane (bilayer curvature strain). It expands the interfacial area and disorders the acyl chains.

There are, however, characteristic differences between the effects of surfactin and classical detergents on membrane structure. The acyl chains of the lipid are tilted by surfactin. The peptidic part of the surfactin molecule inserts into the hydrophobic interface of the membrane, whereas detergent headgroups are localized in the headgroup region. The charged residues are important for antibiotic activity by leading to a

sufficient solubility in the aqueous medium and by hindering a flip-flop of surfactin to the inner membrane leaflet (thus creating an imbalance between the two leaflets).

It must be noted that the same key effects (strong anchoring force, deep insertion of a bulky moiety into the membrane interface, and charge) also apply to other cyclic or α-helical antibiotic peptides. The free energy gain of membrane-assisted helix formation[54] would further add to the anchoring force. Hence, these molecules will also possess very strong detergent-like activity to permeabilize membranes. Whether this effect or another (such as peptide self-association to a pore) accounts for antibiotic activity depends on which of the mechanisms works at lower peptide concentration.

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References and Notes

(1) *Biochim. Biophys. Acta* **1999**, *1462*.
(2) Lohner, K.; Epand, R. *Adv. Biophys. Chem.* **1997**, *6*, 53.
(3) Matsuzaki, K. *Biochim. Biophys. Acta* **1999**, *1462*, 1.
(4) Yang, L.; Weiss, T. M.; Lehrer, R. I.; Huang, H. W. *Biophys. J.* **2000**, *79*, 2002.
(5) Wiese, A.; Munstermann, M.; Gutsmann, T.; Lindner, B.; Kawahara, K.; Zahringer, U.; Seydel, U. *J. Membr. Biol.* **1998**, *162*, 127.
(6) Lohner, K.; Prenner, E. J. *Biochim. Biophys. Acta* **1999**, *1462*, 141.
(7) Bechinger, B. *Biochim. Biophys. Acta* **1999**, *1462*, 157.
(8) Heerklotz, H.; Seelig, J. *Biophys. J.* **2001**, *81*, 1547.
(9) Ladokhin, A. S.; White, S. H. *Biochim. Biophys. Acta* **2001**, *1514*, 253.
(10) Arima, K.; Kakinuma, A.; Tamura, G. *Biochem. Biophys. Res. Commun.* **1968**, *31*, 488.
(11) Kracht, M.; Rokos, H.; Ozel, M.; Kowall, M.; Pauli, G.; Vater, J. *J. Antibiot.* **1999**, *52*, 613.
(12) Vollenbroich, D.; Ozel, M.; Vater, J.; Kamp, R. M.; Pauli, G. *Biologicals* **1997**, *25*, 289.
(13) Vollenbroich, D.; Pauli, G.; Ozel, M.; Vater, J. *Appl. Environ. Microbiol.* **1997**, *63*, 44.
(14) Beven, L.; Wroblewski, H. *Res. Microbiol.* **1997**, *148*, 163.
(15) Kameda, Y.; Oira, S.; Matsui, K.; Kanatomo, S.; Hase, T. *Chem. Pharm. Bull.* **1974**, *22*, 938.
(16) Otten, D.; Lobbecke, L.; Beyer, K. *Biophys. J.* **1995**, *68*, 584.
(17) Otten, D.; Brown, M. F.; Beyer, K. *J. Phys. Chem. B* **2000**, *104*, 12119.
(18) Sheetz, M. P.; Singer, S. J. *Proc. Natl. Acad. Sci. U.S.A* **1974**, *71*, 4457.
(19) Heerklotz, H. *Biophys. J.* **2001**, *81*, 184.
(20) Kragh-Hansen, U.; le Maire, M.; Moller, J. V. *Biophys. J.* **1998**, *75*, 2932.
(21) Matsuzaki, K.; Sugishita, K.; Ishibe, N.; Ueha, M.; Nakata, S.; Miyajima, K.; Epand, R. M. *Biochemistry* **1998**, *37*, 11856.
(22) Hallock, K. J.; Lee, D. K.; Ramamoorthy, A. *Biophys. J.* **2003**, *84*, 3052.
(23) Hristova, K.; Wimley, W. C.; Mishra, V. K.; Anantharamiah, G. M.; Segrest, J. P.; White, S. H. *J. Mol. Biol.* **1999**, *290*, 99.
(24) Matsuzaki, K.; Murase, O.; Tokuda, H.; Funakoshi, S.; Fujii, N.; Miyajima, K. *Biochemistry* **1994**, *33*, 3342.
(25) Bonmatin, J. M.; Genest, M.; Petit, M. C.; Gincel, E.; Simorre, J. P.; Cornet, B.; Gallet, X.; Caille, A.; Labbe, H.; Vovelle, F.; Ptak, M. *Biochimie* **1992**, *74*, 825.
(26) Deleu, M.; Bouffioux, O.; Razafindralambo, H.; Paquot, M.; Hbid, C.; Thonart, P.; Jacques, P.; Brasseur, R. *Langmuir* **2003**, *19*, 3377.
(27) MPEx. http://blanco.biomol.uci.edu/mpex/.
(28) Wimley, W. C.; White, S. H. *Nat. Struct. Biol.* **1996**, *3*, 842.
(29) Grau, A.; Gomez Fernandez, J. C.; Peypoux, F.; Ortiz, A. *Biochim. Biophys. Acta* **1999**, *1418*, 307.
(30) Seelig, A.; Seelig, J. *Biochim. Biophys. Acta* **1975**, *406*, 1.
(31) Buldt, G.; Gally, H. U.; Seelig, A.; Seelig, J.; Zaccai, G. *Nature* **1978**, *271*, 182.
(32) Buldt, G.; Gally, H. U.; Seelig, J.; Zaccai, G. *J. Mol. Biol.* **1979**, *134*, 673.
(33) Seelig, J.; Macdonald, P. M.; Scherer, P. G. *Biochemistry* **1987**, *26*, 7535.
(34) Macdonald, P. M.; Leisen, J.; Marassi, F. M. *Biochemistry* **1991**, *30*, 3558.
(35) Thurmond, R. L.; Otten, D.; Brown, M. F.; Beyer, K. *J. Phys. Chem.* **1994**, *98*, 972.
(36) Wenk, M. R.; Alt, T.; Seelig, A.; Seelig, J. *Biophys. J.* **1997**, *72*, TH331.
(37) Klose, G.; Madler, B.; Schafer, H.; Schneider, K. P. *J. Phys. Chem. B* **1999**, *103*, 3022.
(38) Seelig, J.; Waespe-Sarcevic, N. *Biochemistry* **1978**, *17*, 3310.
(39) Gally, H. U.; Niederberger, W.; Seelig, J. *Biochemistry* **1975**, *14*, 3647.
(40) Heerklotz, H.; Seelig, J. *Biophys. J.* **2000**, *78*, 2435.
(41) Davis, J. H.; Jeffrey, K. R.; Bloom, M.; Valic, M. I.; Higgs, T. P. *Chem. Phys. Lett.* **1976**, *42*, 390.
(42) Wenk, M. R.; Seelig, J. *J. Phys. Chem. B* **1997**, *101*, 5224.
(43) Heerklotz, H.; Binder, H.; Lantzsch, G.; Klose, G.; Blume, A. *J. Phys. Chem. B* **1997**, *101*, 639.
(44) Edwards, K.; Almgren, M. *J. Colloid Interface Sci.* **1991**, *147*, 1.
(45) Heerklotz, H.; Lantzsch, G.; Binder, H.; Klose, G.; Blume, A. *J. Phys. Chem.* **1996**, *100*, 6764.
(46) Lantzsch, G.; Binder, H.; Heerklotz, H.; Wendling, M.; Klose, G. *Biophys. Chem.* **1996**, *58*, 289.
(47) Isrealachvili, J. N. *Intermolecular and Surface Forces*, 2nd ed.; Academic Press: London, 1991.
(48) Seelig, A.; Seelig, J. Membrane Structure. In *Encyclopedia of Physical Science and Technology*, 3rd ed.; Academic Press: San Diego, CA, 2002; Vol. 9, p 355.
(49) Scherer, P. G.; Seelig, J. *Biochemistry* **1989**, *28*, 7720.
(50) Schote, U.; Ganz, P.; Fahr, A.; Seelig, J. *J. Pharm. Sci.* **2002**, *91*, 856.
(51) Seelig, A.; Seelig, J. *Biochemistry* **1974**, *13*, 4839.
(52) Seelig, J. *Q. Rev. Biophys.* **1977**, *10*, 353.
(53) Zaccai, G.; Buldt, G.; Seelig, A.; Seelig, J. *J. Mol. Biol.* **1979**, *134*, 693.
(54) Wieprecht, T.; Apostolov, O.; Beyermann, M.; Seelig, J. *J. Mol. Biol.* **1999**, *294*, 785.