Detergent-Like Action of the Antibiotic Peptide Surfactin on Lipid Membranes

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ABSTRACT Surfactin is a bacterial lipopeptide with powerful surfactant-like properties. High-sensitivity isothermal titration calorimetry was used to study the self association and membrane partitioning of surfactin. The critical micellar concentration (CMC), was 7.5 μM, the heat of micellization was endothermic with $\Delta H_{Su}^{w\rightarrow m} = +4.0$ kcal/mol, and the free energy of micellization $\Delta G_{Su}^{0,w\rightarrow m} = -9.3$ kcal/mol (25°C; 100 mM NaCl; 10 mM TRIS, 1 mM EDTA; pH 8.5). The specific heat capacity of micellization was deduced from temperature dependence of $\Delta H_{Su}^{w\rightarrow m}$ as $\Delta C_P^{w\rightarrow m} = -250 \pm 10$ cal/(mol·K). The data can be explained by combining the hydrophobicity of the fatty acyl chain with that of the hydrophobic amino acids. The membrane partition equilibrium was studied using small (30 nm) and large (100 nm) unilamellar POPC vesicles. At 25°C, the partition coefficient, K, was $(2.2 \pm 0.2) \times 10^4$ M^{-1} for large vesicles leading to a free energy of $\Delta G_{Su}^{0,w\rightarrow b} = -8.3$ kcal/mol. The partition enthalpy was again endothermic, with $\Delta H_{Su}^{w\rightarrow b} = 9 \pm 1$ kcal/mol. The strong preference of surfactin for micelle formation over membrane insertion explains the high membrane-destabilizing activity of the peptide. For surfactin and a variety of non-ionic detergents, the surfactant-to-lipid ratio, inducing membrane solubilization, R_b^{sat}, can be predicted by the simple relationship $R_b^{sat} \approx K \cdot \mathrm{CMC}$.

INTRODUCTION

The growing resistance of bacteria against conventional antibiotics has led to an intense search for new types of antibiotics such as antibiotic peptides. Among these, surfactin is a detergent-like lipopeptide produced by *Bacillus subtilis* (Arima et al., 1968) reducing the surface tension of water from 72 mN/m to ~30 mN/m at concentrations of ~10 μM (Ishigami et al., 1995; Peypoux et al., 1999). Surfactin consists of a heptapeptide headgroup with the sequence Glu-Leu-D-Leu-Val-Asp-D-Leu-Leu closed to a lactone ring by a $C_{14\text{-}15}$ β-hydroxy fatty acid. The peptide ring adopts a "horse-saddle" structure in solution with the two charged residues forming a "claw," which is a potential binding site for divalent cations (Bonmatin et al., 1992). On the opposite side of the ring, the fatty acyl chain may extend into a micellar structure or into a lipid bilayer. Surfactin has a critical micellar concentration (CMC) of 9.4 μM in 200 mM $NaHCO_3$ at pH 8.7 (Ishigami et al., 1995) and forms rod-like micelles with an aggregation number of ~170. The pK_a of aggregated surfactin is about 6 (Maget-Dana et al., 1992).

A variety of important applications and physiological activities have been proposed for surfactin. Surfactin could play a physiological role by increasing the bioavailability of water-insoluble substrates and by regulating the attachment/detachment of microorganisms to and from surfaces (Rosenberg and Ron, 1999). Surfactin has hemolytic (Kracht et al., 1999), antiviral (Vollenbroich et al., 1997a; Kracht et al., 1999), antibacterial (Vollenbroich et al., 1997b; Beven and Wroblewski, 1997), and antitumor (Kameda et al., 1974) properties. These observations have attracted considerable interest because they may all be related to the effect of surfactin on the lipid part of the biological membrane. The application of surfactin as a strong, biodegradable detergent for technical and household purposes can also be envisaged but would require much cheaper production methods (Rosenberg and Ron, 1999).

EXPERIMENTAL

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids (Birmingham, AL). Surfactin (approx. 98% purity) was from Sigma (St. Louis, MO). The substances were used without further purification. All measurements were made in buffer (100 mM NaCl, 10 mM TRIS, 1 mM EDTA; pH 8.5).

POPC was dried from a chloroform/methanol solution by a gentle stream of nitrogen and kept under vacuum over night. After addition of buffer, the samples were vortexed and subject to five freeze/thaw cycles. Finally, large unilamellar vesicles (LUV) of POPC with a diameter of ~100 nm were prepared by extrusion through two stacked Nuclepore polycarbonate membranes of 100-nm pore size (MacDonald et al., 1991). Small unilamellar vesicles (SUV) with a diameter of 30 nm were prepared by ultrasound sonication.

Isothermal titration calorimetry (ITC) was performed with a VP calorimeter (Microcal, Northampton, MA) (Chellani, 1999). The mixing cell had a volume of 1.4 mL. Critical micellar concentrations were determined by calorimetric dilution experiments (Kresheck and Hargraves, 1974; Olofsson, 1985; Paula et al., 1995; Kresheck, 1998). Briefly, the injection syringe is filled with a micellar solu-

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tion of surfactin, typically at a concentration 20 times larger than the CMC. The injection of the micellar solution into the calorimeter cell, containing only buffer, leads to a 100–200-fold dilution of surfactin, and the resulting surfactin concentration in the cell is distinctly below the CMC. The injected micelles disintegrate into monomers, a process accompanied by the consumption or release of heat. The heat of demicellization is measured by the instrument using a power-compensation feedback. Each injection increases the surfactin concentration in the calorimeter cell. As the surfactin concentration approaches the CMC, the injected micelles are no longer dissolved and the heat of demicellization decreases and finally becomes zero. Typically, demicellization occurs over a rather broad concentration range. The CMC is defined as the midpoint of the titration curve, i.e., the first derivative of the heat of titration has its maximum at this concentration.

Isothermal titration calorimetry was also used to measure the partitioning of surfactin into the bilayer membrane. In this type of experiment, the calorimeter cell is filled with a peptide solution below the CMC, typically 5 μM. The syringe is filled with a suspension of unilamellar POPC vesicles at a lipid concentration of 2 mM, and a series of injections is performed ($V_{inj} \sim$ 3–10 μL). At each lipid injection, free surfactin partitions into the bilayer membrane and the corresponding heat of reaction is measured. Obviously, the heats of reaction become smaller during the course of the titration as less and less peptide remains free in solution. Integration of the calorimeter peaks yields the heats δh_i, which are plotted against the lipid concentration in the cell. The evaluation of such data sets has been explained elsewhere (Seelig, 1997; Heerklotz and Seelig, 2000a,b). The heat measured after the ith injection, δh_i, depends on the molar amount of bound surfactin, $\delta n^{(i)}_{Su,b}$, and the standard heat of reaction, $\Delta H^{w\rightarrow b}_{Su}$ ($w\rightarrow b$ denotes the transition of surfactin from the aqueous phase w to the bilayer b),

$$\delta h_i = \delta n^{(i)}_{Su,b} \cdot \Delta H^{w\rightarrow b}_{Su}. \quad (1)$$

The molar ratio, R_b, of surfactin bound to lipid is

$$R_b = n_{Su,b}/n^0_L = C_{Su,b}/C^0_L, \quad (2)$$

where $n_{Su,b}$ is the molar amount of bound surfactin and n^0_L the total lipid in the calorimeter cell. $C_{Su,b}$ and C^0_L are the corresponding concentrations. The total surfactin concentration in the cell, C^0_{Su}, remains constant,

$$C^0_{Su} = C_{Su,b} + C_{Su,f}, \quad (3)$$

and is divided into bound peptide, $C_{Su,b}$, and free peptide, $C_{Su,f}$. The experimental data show that surfactin binding to the lipid membrane can be described by a partition equilibrium of the form

$$R_b = KC_{Su,f}, \quad (4)$$

i.e., the surfactin-to-lipid ratio in the membrane, R_b, is linearly proportional to the free surfactin concentration $C_{Su,f}$ (cf. Lasch, 1995; Heerklotz and Seelig, 2000b, for a discussion of partitioning models). Taking into account Eqs. 2 and 3, Eq. 4 can also be written as

$$C_{Su,b} = C^0_{Su}\frac{KC^0_L}{1 + KC^0_L}, \quad (5)$$

$$n_{Su,b} = C^0_{Su}V_{cell}\frac{KC^0_L}{1 + KC^0_L}. \quad (6)$$

The incremental increase of bound surfactin, $\delta n_{Su,b}$, upon injection of δn^0_L moles of lipid is obtained as the first derivative of Eq. 6,

$$\delta n_{Su,b} = \frac{K \cdot C^0_{Su}}{(1 + K \cdot C^0_L)^2} \cdot \delta n^0_L. \quad (7)$$

The insertion of Eq. 7 into 1 makes it possible to fit K and $\Delta H^{w\rightarrow b}_{Su}$ to the experimental data,

$$\frac{\delta h_i}{\delta n^0_L} = \frac{K \cdot C^0_{Su}}{(1 + K \cdot C^0_L)^2} \cdot \Delta H^{w\rightarrow b}_{Su} + q_{dil}. \quad (8)$$

The heat of dilution of the injected vesicle suspension, q_{dil}, can be measured in a separate experiment or adjusted as a third fit parameter.

Surfactin carries two negatively charged amino acid side chains (glu-1, asp-5) and electrostatic attraction or repulsion cannot be ignored a priori. A treatment of this problem is possible by using the Gouy–Chapman theory (Seelig, 1997). For surfactin, the application of the Gouy–Chapman theory leads to the conclusion that the total effective charge seen at the membrane surface is, at most, $z_p = -0.5$. With $z_p = -0.5$, the fit to the experimental data is as good as for $z_p = 0$ (within the accuracy of the measurements) but the binding constants are ~10–20% higher. For the present study, the partition model without electrostatics appears to be a sufficient approximation.

RESULTS

Critical micellar concentration

Figure 1 *A* shows the results for the injection of a 0.5-mM surfactin solution into buffer at 15°C. The calorimeter cell has a volume of V_{cell} = 1.4037 mL, and each injection (V_{inj} = 4 μL) is accompanied by an exothermic heat of reaction caused by the disintegration of surfactin micelles. As the titration proceeds, the surfactin concentration in the calorimeter cell increases and, finally, micelles are no longer dissolved. The magnitude of the titration peaks decreases correspondingly. Integration of the power peaks yields the heat of reaction, δh_i. Normalization by the injected mole number δn^0_L (δn^0_L = 2 nmol in Fig. 1) leads to the molar heat of demicellization. In Fig. 1 *B*, $\delta h_i/\delta n^0_L$ is

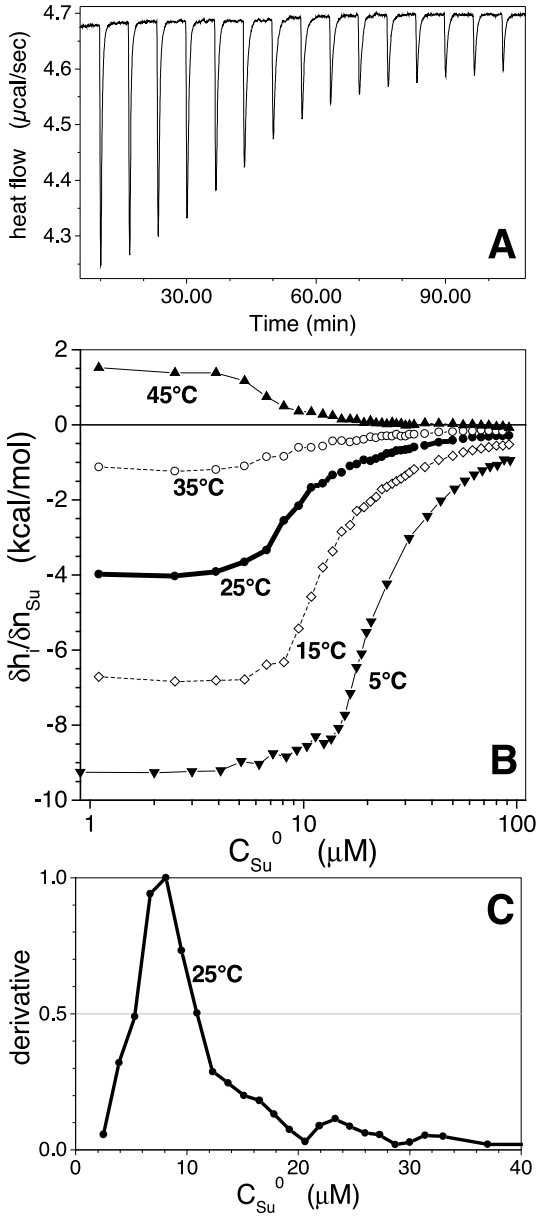

FIGURE 1 ITC demicellization experiments. (*A*). Raw data for a set of 7-μL injections of a 0.5-mM surfactin solution into buffer (10 mM Tris, 100 mM NaCl, pH 8.5) at 15°C, showing the heat flow per injection. (*B*) Heats of injection, δh_i, per mole of surfactin injected, δn_i, versus the surfactin concentration in the cell, C_S^0 at 5°C (▼), 15°C (◇), 25°C (*bold line*, ●), 35°C (○), and 45°C (▲). (*C*) Normalized first derivative of the 25°C curve of (*B*). The maximum is defined as the CMC.

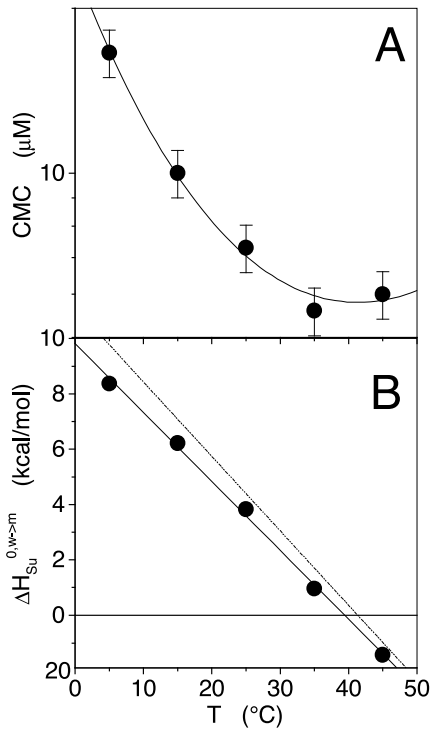

FIGURE 2 (*A, log scale*) the critical micelle concentration, CMC, and (*B*) Temperature dependence of the enthalpy of micelle formation $\Delta H_{Su}^{w\rightarrow m}$. The data are taken from Fig. 1. The slope of the linear fit in (*B*) is $\Delta C_P^{w\rightarrow m} = -(250 \pm 10)$ cal/(mol·K) and the isocaloric temperature is 41°C (*solid line*). The fit of log(CMC) versus T (*solid line* in *A*, cf. Kresheck, 1998) predicts the van't Hoff enthalpy indicated by the dash/dot line in (*B*).

plotted as a function of the total surfactin concentration. Titrations were performed at temperatures in the range of 5 to 45°C and all titration curves follow a sigmoidal pattern. Figure 1 *C* displays the first derivative of the titration curve measured at 25°C. The derivative curve has a maximum at 7.5 μM, which is defined as the CMC (Paula et al., 1995). Figure 1 further demonstrates that the disintegration of surfactin micelles extends over a rather broad concentration range.

The variation of the CMC with temperature is shown in Fig. 2 *A*. The CMC is large at low temperatures, goes through a minimum at ~40°C and increases again beyond this temperature. At the minimum, the enthalpy of micelle formation/demicellization must be zero. The standard enthalpy of micelle formation, $\Delta H_{Su}^{w\rightarrow m}$ (w, water; m, micelle; Su, surfactin), is identical in magnitude with the experimentally measured heat of demicellization but has the opposite sign. $\Delta H_{Su}^{w\rightarrow m}$ can be read off directly from the initial plateau region of Fig. 1 *B* (correcting for the heat of dilution measured above the CMC). The corresponding results are plotted as a function of temperature in Fig. 2 *B*.

At low temperatures, micelle formation is enthalpically unfavorable with an endothermic reaction enthalpy of +9.3 kcal/mol at 5°C. $\Delta H_{Su}^{w\rightarrow m}$ decreases linearly with increasing temperature. It becomes zero at ~40°C and is exothermic at higher temperatures. From the slope of the straight line, the change in the molar heat capacity upon micelle formation is determined as $\Delta C_p^{w\rightarrow m} = (-250 \pm 10)$ cal/(mol·K). As a second approach, the temperature dependence of the CMC can be used to determine $\Delta H_{Su}^{w\rightarrow m}$ (cf. Kresheck, 1998) and, in turn, the corresponding $\Delta C_p^{w\rightarrow m} = -(270 \pm 50)$ cal/(mol·K) (details not shown). The results are in agreement with the direct calorimetric measurement but are less pre-

TABLE 1 Thermodynamic parameters for micelle formation and membrane partitioning of surfactin

Micelle formation	CMC* (μM)	ΔG^0 (kcal/mol)	ΔH^* (kcal/mol)	$T\Delta S$ (kcal/mol)
5°C	16.6	−8.3	8.4	17
15°C	10.0	−8.9	6.2	15
25°C	7.5	−9.4	4.0	13
35°C	5.6	−9.9	1.0	11
45°C	6.0	−10.1	−1.4	9
Membrane partitioning	K (mM^{-1})			
LUV, 25°C (d = 100 nm)	22 ± 2	−8.3	9 ± 1	17
SUV, 25°C (d = 30 nm)	50 ± 15	−8.8	3.2 ± 0.5	12
SUV, 40°C (d = 30 nm)	30 ± 20	−6.4	1.6 ± 0.5	8

*The precision of ITC measurements is generally better than 10%. The error can be larger if the standard reaction enthalpy is close to zero and the measured δh_i are very small.

cise. The ITC measurements are summarized in numerical form in Table 1.

Membrane partitioning of surfactin

The thermodynamics of surfactin binding to lipid vesicles was determined by lipid-into-surfactin titrations. In the experiment shown in Fig. 3 *A*, a 7.5-μM surfactin solution (in buffer, pH 8.5) in the calorimeter cell is titrated with POPC lipid vesicles (~100-nm diameter, also in buffer at pH 8.5). Each titration peak corrsponds to the injection of 7.5 μL of a 2-mM lipid suspension. The partitioning of surfactin into the lipid bilayer is endothermic, and the heat of reaction decreases gradually as less and less surfactin is available for binding. After the very first injection, all further titration peaks exhibit a biphasic behavior, consisting of a fast endothermic reaction in the time range of seconds followed by a slower, again endothermic process in the minute time range. The first process is surfactin binding to the outer vesicle surface, the second is tentatively assigned to a slow translocation of the molecule to the inner membrane followed by additional surfactin binding. Similar experiments were performed at other surfactin concentrations. Integration of the heat flow peaks in Fig. 3 *A* yields the heats of reaction, h_i, which can be normalized with respect to the injected mole number of lipids, δn_L. Figure 3 *B* shows the variation of the normalized heat of reaction versus the total lipid concentration in the cell for three different surfactin solutions.

The solid lines are the best three-parameter fits to the data according to the partition model described above (apart from the first injection into 10 μM surfactin, which yields $R_b > R_b^{sat}$ and was excluded, cf. Fig. 3, *B* and *C*). The partition constant is $K = (22 \pm 2) \cdot 10^3\ M^{-1}$, the partition enthalpy is $\Delta H_{Su}^{w\to b} = (9 \pm 1)$ kcal/mol, and the heat of dilution is $q_{dil} = -0.23$ kcal/mol (at 25°C). The analysis is

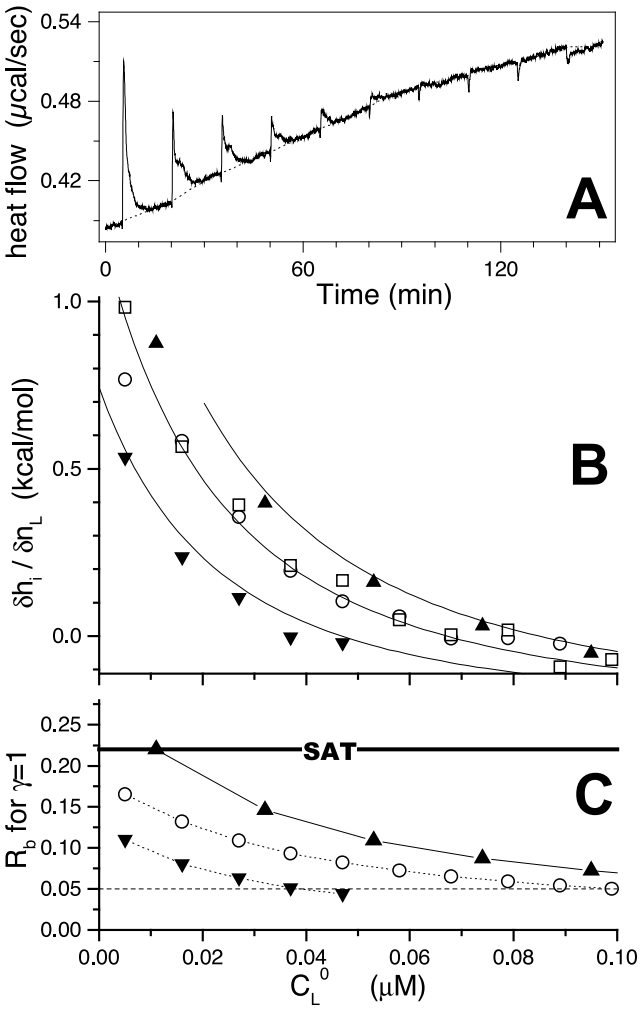

FIGURE 3 Partitioning of surfactin into 100-nm POPC vesicles. (*A*) ITC experiment. Injection of 7.5-μL aliquots of lipid vesicles (2 nm POPC) into a 7.5-μM surfactin solution. (*B*) The heat of reaction, δh_i, divided by the molar amount of injected lipid is plotted versus the lipid concentration in the calorimeter cell, C_L^0. The different symbols refer to four different experiments with the following surfactin concentrations 5 μM (▼), 7.5 μM (○, □), 10 μM (▲). The solid lines are theoretical fits according to Eq. 5 with the following parameters $K = 2.2 \times 10^4\ M^{-1}$, $\Delta H_{Su}^{w\to b} = 9.0$ kcal/mol, $q_{dil} = -0.23$ kcal/mol. (*C*) Variation of the surfactin (bound)-to-lipid ratio, R_b as a function of the lipid concentration in the calorimeter cell. The solid line *SAT* denotes the limiting detergent-to-lipid ratio at which membrane micellization occurs.

based on the assumption that surfactin equilibrates between the outer and inner lipid layer within 15 min under the experimental conditions chosen ($R_b > 0.05$). The same parameters may then be used to calculate the extent of binding, R_b, defined as the molar ratio of bound detergent to total lipid (inside plus outside) as shown in Fig. 3 *C*. The R_b parameter is high during the initial injections and increases with increasing lipid content.

If the surfactin concentration in the calorimeter cell reaches the CMC, the injected phospholipid vesicles are no longer stable but disintegrate to form mixed micelles. The

limiting surfactin-to-lipid ratio, R_b^{sat}, at which this process is initiated, can also be determined with titration calorimetry (Heerklotz and Seelig, 2000b). A limiting value of $R_b^{sat} = 0.22$ was observed for surfactin (data not shown).

DISCUSSION

Micelle formation

The CMC value of 7.5 μM (at 25°C) as determined by ITC is in broad agreement with surface tension measurements yielding 9.4 μM (100 mM $NaHCO_3$, pH 8.7) (Ishigami et al., 1995). The CMC determination by the film balance method is biased toward the endpoint of the titration when a constant surface tension is reached. The corresponding detergent concentration is thus expected to be higher than that observed with ITC, where the midpoint of the monomer $\rightleftarrows$ micelle transition is determined. In contrast, the use of hydrophobic dyes in 10 mM citrate buffer (pH 10, no salt) yields a much higher CMC of 300 μM (Maget-Dana and Ptak, 1992) because electrostatic repulsion between the peptide molecules is stronger at lower ionic strength.

The aggregation of n anionic surfactants to form micelles can be accompanied by the binding of m cations and is represented by the scheme (cf. Hiemenz, 1986),

$$nS^- + mM^+ \rightleftarrows (S_n^- M_m^+)^{z-},$$

in which $z = n - m$ is the net charge of the micelle. The free energy of micelle formation is (Hiemenz, 1986)

$$\Delta G^{0,w\rightarrow m} = RT\left(1 + \frac{m}{n}\right)\ln(\mathrm{CMC}/C_w). \qquad (9)$$

For neutral surfactants or under conditions where no cation M^+ associates with the micelle (high salt), $m = 0$ and Eq. 9 simplifies to

$$\Delta G_{Su}^{0,w\rightarrow m} = RT\ln(\mathrm{CMC}/C_w). \qquad (10)$$

The same result can be derived by calculating the standard free energy change for the addition of a single detergent molecule to an aggregate having the size most probable at the CMC (Emerson and Holtzer, 1965). The factor $C_w = 55.5$ M denotes the concentration of water. CMC/C_w thus represents the mole fraction of surfactant in the aqueous phase (cf. Tanford, 1980). The superscript w→m indicates the transition of surfactin (Su) from water (w) into the micelle (m). For CMC = 7.5 μM, the free energy is $\Delta G_{Su}^{0,w\rightarrow m} = -9.3$ kcal/mol. The enthalpy of micellization is $\Delta H_{Su}^{w\rightarrow m} = +4.0$ kcal/mol at 25°C. The driving force for micellization is thus the large positive entropy term $T\Delta S_{Su}^{w\rightarrow m} = 13.3$ kcal/mol. As shown in Fig. 2 *B*, micelle formation also entails a large change in the specific heat capacity of $\Delta C_P^{w\rightarrow m} = (-250 \pm 10)$ cal/(mol·K).

The positive entropy and the large heat-capacity change of micellization are commonly explained by the so-called hydrophobic effect. In this model, the insertion of a nonpolar substance into an aqueous environment is assumed to cause an ordering of the water molecules around the nonpolar moiety, and the large hydration shell is responsible for the large change in C_p. The association of nonpolar molecules to form a micelle reduces the total hydration shell and produces a large negative $\Delta C_p^{w\rightarrow m}$. This rather simple model has been modified (Privalov and Gill, 1989) and criticized (Muller, 1990, 1992) and alternative views have been proposed (Muller, 1990; Cooper, 2000). For peptides, it has become clear that the specific heat capacity also has contributions of opposite sign from polar moieties (Spolar et al., 1992; Baker and Murphy, 1998).

The following discussion should thus be considered as a first-order approximation only. Solubility studies of hydrocarbon molecules and hydrophobic peptides have led to an empirical, linear relationship between the specific heat capacity and the number of hydrophobic hydrogen atoms, n_H, moved from the apolar phase into water,

$$\Delta C_p^{w\rightarrow m} = \alpha \cdot n_H. \qquad (11)$$

The factor α is 7.9 cal/(mol·K) for small hydrocarbons, and 6.7 cal/(mol·K) for peptide partitioning and micelle formation (Baker and Murphy, 1998; Heerklotz and Epand, 2001). Based on the surfactin heat capacity of $\Delta C_p^{w\rightarrow m} = -250$ cal/(mol·K), the number of nonpolar hydrogens buried during micelle formation is estimated between 32 and 37. The major contribution comes from the fatty acyl chain (C_{14}-C_{15} β-hydroxy) with 23 to 25 nonpolar H-atoms, the remaining 7–14 nonpolar H-atoms must be located in the peptide ring. This is supported by the NMR structure of surfactin in solution with residues D-leu-3, val-4, and leu-7 facing the hydrophobic side of surfactin (Bonmatin et al., 1992; Peypoux et al., 1999).

The contribution of a hydrophobic CH_2 group to the standard free energy of micelle formation has also been measured and is ~ -0.7 kcal/mole CH_2 (Tanford, 1980; Clint, 1992). For a fatty acyl chain with 12 free CH_2/CH_3 segments (C_{15} β-hydroxy), the expected free energy of the hydrophobic effect is -8.8 kcal/mol, which is close to the experimental value of $\Delta G_{Su}^{0,w\rightarrow m} = -9.3$ kcal/mol. The contribution of the peptide ring to the standard free energy of micelle formation is thus small and slightly favorable. Hydrophobic (estimated to be -2.5 to -5 kcal/mol) and hydrophilic (electrostatic) components appear to balance each other essentially as far as the peptide head group is concerned.

Membrane partitioning

The present ITC experiments provide the first measurement of the membrane partition coefficient of surfactin. For unilamellar POPC vesicles with a diameter $d \sim 100$ nm the partition coefficient from water (w) to the bilayer (b) is $K =$

$2.2 \times 10^4\ M^{-1}$ at 25°C, assuming an equilibration of surfactin between the outer and inner monolayer. The partition enthalpy is $\Delta H_{Su}^{w\to b} = 9 \pm 1$ kcal/mol at 25°C. For small unilamellar vesicles with $d = 30$ nm, the partition coefficient was found to be slightly larger with $(5 \pm 1.5) \times 10^4\ M^{-1}$ but the enthalpy was smaller with $\Delta H_{Su}^{w\to b} = 3.2 \pm 0.5$ kcal/mol at 25°C. An increase in temperature to 40°C yields $K = (3 \pm 2) \times 10^4\ M^{-1}$ and $\Delta H_{Su}^{w\to b} = 1.6 \pm 0.5$ kcal/mol for 30 nm vesicles. The specific heat capacity is only $\Delta C_P^{w\to b} = -(110 \pm 70)$ cal/mol · K.

The standard free energy of surfactin incorporation into the lipid bilayer is

$$\Delta G_{Su}^{w\to b} = -RT \ln(KC_w), \tag{12}$$

yielding a value of −8.3 kcal/mol at 25°C for LUVs. With $\Delta H_{Su}^{w\to b} = +9$ kcal/mol the entropy term can be calculated as $T\Delta S_{Su}^{w\to b} = +17.3$ kcal/mol, which is larger by ~4 kcal/mol than that obtained for micelle formation.

The distinct difference between the enthalpies of surfactin partitioning into small and large unilamellar vesicles ($\delta\Delta H = 5.8$ kcal/mol) is not unique. Similar results have been observed before for a number of peptidic substances. One of the best documented cases is the membrane partition equilibrium of the cyclic peptide octreotide where the enthalpy increases from −7.0 kcal for 30-nm vesicles to +1.0 kcal for 100-nm vesicles ($\delta\Delta H = 8.0$ kcal/mol) (Beschiaschvili and Seelig, 1992). A tentative explanation has been given on the basis of the inner membrane pressure. Analogous results have been published for magainin 2 amide (Wieprecht et al., 2000b) and for apo-lipoprotein A-I model peptides (Gazzara et al., 1997). It was also found that nonpeptidic drug molecules and other amphipathic molecules partition into sonicated 30 nm vesicles with a large negative enthalpy (Binford and Wadso, 1984; Seelig and Ganz, 1991) giving rise to the idea of a nonclassical hydrophobic effect. In contrast, partitioning of the nonionic surfactant octyl glucoside into lipid vesicles shows only a small increase in ΔH (Wenk et al., 1997).

Membrane destabilization

The free energies of micellization and membrane partitioning are $RT \ln(\mathrm{CMC}/C_w)$ and $-RT \ln(KC_w)$. We have shown recently for a series of non-ionic detergents that an approximately linear relationship exists between the free energies of micellization and partitioning (Heerklotz and Seelig, 2000a). Figure 4 reproduces the original data and also includes the surfactin result. The CMC of surfactin is about two orders of magnitude smaller than those of most other detergents. Nevertheless, the surfactin data is in broad agreement with the linear ln CMC versus ln K scheme. It should be noted that surfactin is the only ionic detergent in Fig. 4, which might explain its stronger deviation from the diagonal.

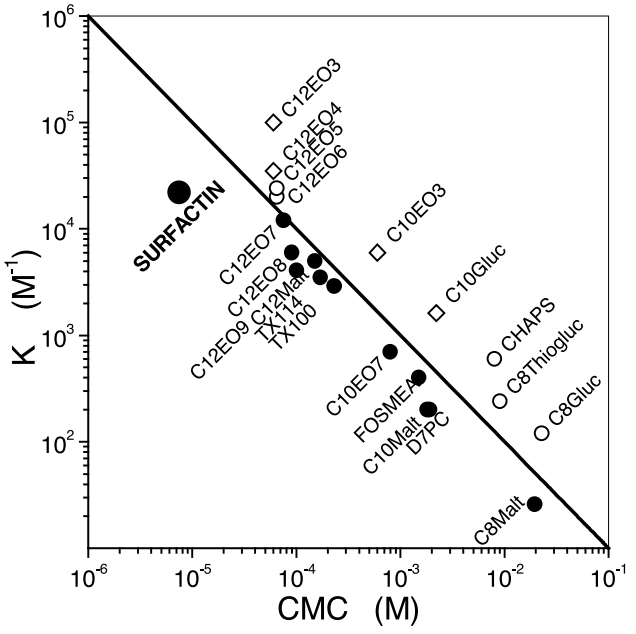

FIGURE 4 Double logarithmic plot of the partition coefficient, K, versus the critical micelle concentration, CMC, of surfactin compared to $C_{12}EO_9$ (nonaethyleneglycol dodecyl ether), FOSMEA (dodecyl phospho-*n*-methylethanolamine) (details not shown) and other non-ionic surfactants as described in Heerklotz and Seelig (2000a). The diagonal line corresponds to $K \cdot \mathrm{CMC} = 1$. An offset from the diagonal to lower K values (i.e., $K \cdot \mathrm{CMC} < 1$) indicates strong membrane destabilization by the respective compound.

Of practical importance (e.g., for the purification of biological membrane components) is the limiting surfactant-to-lipid ratio, R_b^{sat}, which initiates membrane solubilization. For surfactin, this value was determined with ITC as $R_b^{sat} \approx 0.22$ mol surfactin/mol POPC, whereas, for most other detergents, a higher detergent incorporation (>0.6 mol/mol) is required. A given detergent has two competing options: either to penetrate into the lipid bilayer or to self-associate into a micellar structure. Which process is realized, depends on the relative magnitudes of K and CMC and the entropy of mixing. The standard free energy difference between micellization and membrane penetration is $\Delta G_{Su}^{0,b\to m} = \Delta G_{Su}^{0,w\to m} - \Delta G_{Su}^{0,w\to b} = RT \ln(\mathrm{CMC} \cdot K)$. For $\mathrm{CMC} \cdot K < 1$, micellization is preferred; for $\mathrm{CMC} \cdot K > 1$ membrane penetration is favored at low concentration.

This suggests that the saturation limit R_b^{sat} is related to the product $(K \cdot \mathrm{CMC})$ of the two parameters. Figure 5 then shows a plot of R_b^{sat} versus $(K \cdot \mathrm{CMC})$ for the detergents summarized in Fig. 4. A linear relationship is obtained, and linear regression analysis yields $R_b^{sat} = 0.08 + 0.9\ (K \cdot \mathrm{CMC})$ leading to the simplified relationship,

$$R_b^{sat} \approx K \cdot \mathrm{CMC}, \tag{13}$$

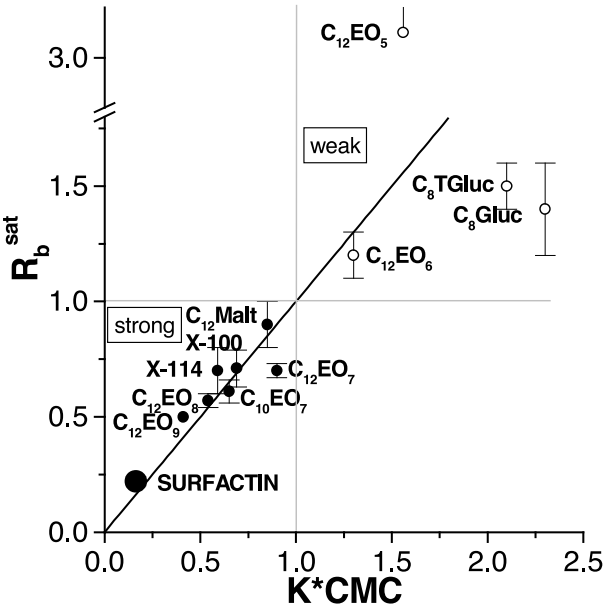

FIGURE 5 Correlation between the surfactant-to-lipid mole ratio in the membrane at the onset of solubilization, R_b^{sat}, and the membrane destabilization specified in terms of $K \cdot$ CMC. Surfactin is compared to non-ionic surfactants as characterized in Heerklotz and Seelig (2000a). The bold line represents $R_b^{sat} = K \cdot$ CMC.

i.e., a straight line of slope 1 through the origin. Comparison of Eq. 13 with 4 implies that

$$C_{Su,f}^{sat} \approx \text{CMC}, \tag{14}$$

i.e., Eq. 13 can be traced back to the fact that solubilization is initiated at a free surfactant concentration, $C_{Su,f}^{sat}$, sufficient to form stable mixed micelles, but slightly below the critical concentration necessary to form pure surfactant micelles, the CMC. Eq. 14 appears to be a "rule-of-thumb" to predict the rupture point of a membrane if the CMC of a detergent and the corresponding membrane partition coefficient are known. Inspection of fig. 5 reveals that detergents may be classified as "strong detergents" if $R_{sat} < 1$ and as "weak detergents" if $R_b^{sat} > 1$. Following this classification, surfactin, with $K \cdot \text{CMC} \approx 0.2$ and $R_b^{sat} = 0.22$, must be considered as one of the strongest detergents in this group, leading to membrane disruption at one of the lowest R_b^{sat} of all detergents considered so far.

Like synthetic, non-ionic detergents the bacterial peptide surfactin binds to zwitterionic membranes driven by entropy but opposed by an endothermic $\Delta H_{Su}^{w\rightarrow b}$ at room temperature because the driving force is the hydrophobic effect. This is in contrast to most antimicrobial peptides produced by eucaryotes, such as magainin 2 amide (Matsuzaki and Seelig, 1995) PGLa (Wieprecht et al., 2000a), or 18A (Spuhler et al., 1994) which are predominantly positively charged and bind selectively to negatively charged, prokaryotic membranes by a combination of electrostatic and hydrophobic forces. Therefore, the basic peptides have a direct, pronounced effect on the lipid head group structure, whereas both surfactin and non-ionic detergents act primarily on the membrane interface and core (cf. also Wenk et al., 1997).

The unique feature of surfactin compared to detergents is its cyclic peptide headgroup, which is not completely hydrophilic but has an amphiphilic nature, with the charged side chains protruding into the water and some apolar residues reaching into the hydrophobic core of the membrane. This additional anchoring contributes distinctly to the much stronger destabilization of the membrane by surfactin.

The biological activity of surfactin is supposedly related to its ability to destabilize and permeabilize membranes already at concentrations far below the onset of micellization. The preference of surfactin for micelles is $RT \ln(K \cdot \text{CMC}) = -1.1$ kcal/mol compared to ~ -0.5 kcal/mol for strong non-ionic detergents (at 0.1 M salt). This is astonishing because micelle formation of surfactin is opposed by a strong electrostatic repulsion between the peptidic head groups of ~3 kcal/mol, which does not occur for non-ionic detergents. Despite this electrostatic effect, surfactin prefers to form micelles rather than to insert into a lamellar structure.

REFERENCES

Arima, K., A. Kakinuma, and G. Tamura. 1968. Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. *Biochem. Biophys. Res. Commun.* 31:488–494.

Baker, B. M., and K. P. Murphy. 1998. Prediction of binding energetics from structure using empirical parametrization. *Methods Enzymol.* 295: 294–314.

Beschiaschvili, G., and J. Seelig. 1992. Peptide binding to lipid bilayers. Nonclassical hydrophobic effect and membrane-induced pK shifts. *Biochemistry.* 31:10044–10053.

Beven, L., and H. Wroblewski. 1997. Effect of natural amphipathic peptides on viability, membrane potential, cell shape and motility of mollicutes. *Res. Microbiol.* 148:163–175.

Binford, J. S., Jr., and I. Wadso. 1984. Calorimetric determination of the partition coefficient for chlorpromazine hydrochloride in aqueous suspensions of dimyristoylphosphatidylcholine vesicles. *J. Biochem. Biophys. Methods.* 9:121–131.

Bonmatin, J. M., M. Genest, M. C. Petit, E. Gincel, J. P. Simorre, B. Cornet, X. Gallet, A. Caille, H. Labbe, F. Vovelle, and M. Ptak. 1992. Progress in multidimensional NMR investigations of peptide and protein 3-D structures in solution. From structure to functional aspects. *Biochimie.* 74:825–836.

Chellani, M. 1999. Isothermal titration calorimetry: biological applications. *Amer. Biotechnol. Lab.* 17:14–18.

Clint, J. H. 1992. Surfactant Aggregation. Blackie & Son, Glasgow, U.K. 115.

Cooper, A. 2000. Heat capacity of hydrogen-bonded networks: an alternative view of protein folding thermodynamics. *Biophys. Chem.* 85: 25–39.

Emerson, M. F., and A. Holtzer. 1965. On the ionic strength dependence of micelle number. *J. Phys. Chem.* 69:3718–3721.

Gazzara, J. A., M. C. Phillips, S. Lund-Katz, M. N. Palgunachari, J. P. Segrest, G. M. Anantharamaiah, W. V. Rodrigueza, and J. W. Snow. 1997. Effect of vesicle size on their interaction with class A amphipathic helical peptides. *J. Lipid Res.* 38:2147–2154.

Heerklotz, H., and R. M. Epand. 2001. The enthalpy of acyl chain packing and the apparent water accessible apolar surface area of phospholipids. *Biophys. J.* 80:271–279.

Heerklotz, H., and J. Seelig. 2000a. Correlation of membrane/water partition coefficients of detergents with the critical micelle concentration. *Biophys. J.* 78:2435–2440.

Heerklotz, H., and J. Seelig. 2000b. Titration calorimetry of surfactant-membrane partitioning and membrane solubilization. *Biochim. Biophys. Acta.* 1508:69–85.

Hiemenz, P. C. 1986. Principles of colloid and surface chemistry. Marcel Dekker Inc., New York.

Ishigami, Y., M. Osman, H. Nakahara, Y. Sano, R. Ishiguro, and M. Matsumoto. 1995. Significance of beta-sheet formation for micellization and surface adsorption of surfactin. *Colloids Surf. B.* 4:341–348.

Kameda, Y., S. Oira, K. Matsui, S. Kanatomo, and T. Hase. 1974. Antitumor activity of *Bacillus natto*. V. Isolation and characterization of surfactin in the culture medium of *Bacillus natto* KMD 2311. *Chem. Pharm. Bull. (Tokyo).* 22:938–944.

Kracht, M., H. Rokos, M. Ozel, M. Kowall, G. Pauli, and J. Vater. 1999. Antiviral and hemolytic activities of surfactin isoforms and their methyl ester derivatives. *J. Antibiot. (Tokyo).* 52:613–619.

Kresheck, G. C. 1998. Comparison of the calorimetric and van't Hoff enthalpy of micelle formation for a nonionic surfactant in H_2O and D_2O solutions from 15 to 40°C. *J. Phys. Chem. B.* 102:6596–6600.

Kresheck, G. C., and W. A. Hargraves. 1974. Thermometric titration studies of the effect of head group, chain length, solvent, and temperature on the thermodynamics of micelle formation. *J. Coll. Interf. Sci.* 48:481–493.

Lasch, J. 1995. Interaction of detergents with lipid vesicles. *Biochim. Biophys. Acta*. 1241:269–292.

MacDonald, R. C., R. I. MacDonald, B. P. Menco, K. Takeshita, N. K. Subbarao, and L. R. Hu. 1991. Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. *Biochim. Biophys. Acta.* 1061: 297–303.

Maget-Dana, R., and M. Ptak. 1992. Interfacial properties of surfactin. *J. Coll. Interf. Sci.* 153:285–291.

Maget-Dana, R., L. Thimon, F. Peypoux, and M. Ptak. 1992. Surfactin/iturin A interactions may explain the synergistic effect of surfactin on the biological properties of iturin A. *Biochimie.* 74:1047–1051.

Matsuzaki, K., and J. Seelig. 1994. NMR study on interactions of an antibiotic peptide, magainin-2, with lipid bilayers. *In* Peptide Chemistry. N. Ohno, editor. Protein Research Foundation, Osaka, Japan. 129–132.

Muller, N. 1990. Search for a realistic view of hydrophobic effects. *Acc. Chem. Res.* 23:23–28.

Muller, N. 1992. Does hydrophobic hydration destabilize protein native structures? *TIBS.* 17:459–463.

Olofsson, G. 1985. Microtitration calorimetric study of the micellization of three poly (oxyethylene) glycol dodecyl ethers. *J. Phys. Chem.* 89: 1473–1477.

Paula, S., W. Süs, J. Tuchtenhagen, and A. Blume. 1995. Thermodynamics of micelle formation as a function of temperature: a high sensitivity titration calorimetry study. *J. Phys. Chem.* 99:11742–11751.

Peypoux, F., J. M. Bonmatin, and J. Wallach. 1999. Recent trends in the biochemistry of surfactin. *Appl. Microbiol. Biotechnol.* 51:553–563.

Privalov, P. L., and S. J. Gill. 1989. The hydrophobic effect: a reappraisal. *Pure Appl. Chem.* 61:1097–1104.

Rosenberg, E., and E. Z. Ron. 1999. High- and low-molecular-mass microbial surfactants. *Appl. Microbiol. Biotechnol.* 52:154–162.

Seelig, J. 1997. Titration calorimetry of lipid–peptide interactions. *Biochim. Biophys. Acta.* 1331:103–116.

Seelig, J., and P. Ganz. 1991. Nonclassical hydrophobic effect in membrane binding equilibria. *Biochemistry.* 30:9354–9359.

Spolar, R. S., J. R. Livingstone, and M. T. Record, Jr. 1992. Use of liquid hydrocarbon and amide transfer data to estimate contributions to thermodynamic functions of protein folding from the removal of nonpolar and polar surface from water. *Biochemistry.* 31:3947–3955.

Spuhler, P., G. M. Anantharamaiah, J. P. Segrest, and J. Seelig. 1994. Binding of apolipoprotein A-I model peptides to lipid bilayers. Measurement of binding isotherms and peptide-lipid headgroup interactions. *J. Biol. Chem.* 269:23904–23910.

Tanford, C. 1980. The Hydrophobic Effect: Formation of Micelles and Biological Membranes. Wiley, New York. 61–77.

Vollenbroich, D., M. Ozel, and J. Vater, R. M. Kamp, and G. Pauli. 1997a. Mechanism of inactivation of enveloped viruses by the biosurfactant surfactin from *Bacillus subtilis*. *Biologicals.* 25:289–297.

Vollenbroich, D., G. Pauli, M. Ozel, and J. Vater. 1997b. Antimycoplasma properties and application in cell culture of surfactin, a lipopeptide antibiotic from *Bacillus subtilis*. *Appl. Environ. Microbiol.* 63:44–49.

Wenk, M. R., T. Alt, A. Seelig, and J. Seelig. 1997. Octyl-β-D-glucopyranoside partitioning into lipid bilayers: thermodynamics of binding and structural changes of the bilayer. *Biophys. J.* 72:1719–1731.

Wieprecht, T., O. Apostolov, M. Beyermann, and J. Seelig. 2000a. Membrane binding and pore formation of the antibacterial peptide PGLa: thermodynamic and mechanistic aspects. *Biochemistry.* 39:442–452.

Wieprecht, T., O. Apostolov, and J. Seelig. 2000b. Binding of the antibacterial peptide magainin 2 amide to small and large unilamellar vesicles. *Biophys. Chem.* 85:187–198.