0273-2289/01/90/0199/$13.00

Lichenysin

A More Efficient Cation Chelator Than Surfactin

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Received November 1, 1999; Revised May 17, 2000; Accepted July 27, 2000

Abstract

The lipopeptide lichenysin (cyclo-[L-Gln1→D-Leu2→L-Leu3→L-Val4→L-Asp5→D-Leu6→L-Ile7-β-OH fatty acid]) produced by *Bacillus licheniformis* structurally resembles surfactin from *Bacillus subtilis*. The main difference is the presence of a glutaminyl residue in position 1 of the peptide sequence in place of glutamic acid in surfactin. This local variation causes significant changes in the properties of the molecule compared to surfactin. Lichenysin has a higher surfactant power, the critical micellar concentration (c.m.c.) being strongly reduced from 220 to 22 μ*M* and a much higher hemolytic activity because 100% hemolysis was observed with only 15 μ*M* instead of 200 μ*M*. Lichenysin is also a better chelating agent because its association constants with Ca^{2+} and Mg^{2+} are increased by a factor of 4 and 16, respectively. This effect is assigned to an increase in the accessibility of the carboxyl group to cations owing to a change in the side chain topology induced by the Glu/Gln exchange. Additionally, the propensity of the lipopeptide for extensive hydrophobic interactions, as illustrated by its low c.m.c., contributes to further stabilization of the cation and an increase in the partitioning of lichenysin into the erythrocyte membrane. Our data support the formation of a lichensyin-Ca^{2+} complex in a molar ratio of 2:1 instead of 1:1 with surfactin, suggesting an intermolecular salt bridge between two lichenysin molecules. Therefore, when Ca^{2+} ions are present in the solution, micellization occurs via a dimer assembly, with a possible long-range effect on the spatial arrangement of the micelles or other supramolecular structures. Finally, among all the surfactin peptidic variants so far known, lichenysin

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is the one for which the three tested activities are the most substantially improved.

Index Entries: Surfactin; lichenysin; biosurfactant; lipopeptide; cation binding; hemolysis.

Introduction

Lichenysin belongs to the family of the lipopeptide biosurfactants produced by *Bacilli* sp. Among them, surfactin is the main representative and its best known member *(1)*. In addition to its surface activity, this compound exhibits various interesting biological properties making it quite attractive for both industrial and biological applications. It inhibits fibrin clot formation *(2)*; exhibits a hypocholesterolemic effect *(3)*, antiviral, antitumoral, and anti-*Mycoplasma* activities *(4–6)*; inhibits enzymes such as cyclic adenosine monophosphate phosphodiesterase *(7)* and phospholipase A_2 *(8)*; interacts with phospholipids; and induces the formation of selective cationic channels in artificial membranes *(9)*. Lichenysin is placed in this family because of its great similarity to surfactin with respect to the bacterial sources and general structural and biosynthetic features. Its structure includes a 3(R)-hydroxy fatty acid ranging from 13 to 15 carbon atoms in the *n*, iso, and *anteiso* configuration, linked to the heptapeptide sequence L-Gln1→L-Leu2→D-Leu3→L-Val4→L-Asp5→D-Leu6→L-Ile7 by an amide bond and to the terminal carboxyl of the L-isoleucine residue by a lactone bond *(10–12)*.

In analogy with surfactin, lichenysin biosynthesis is assumed to be catalyzed nonribosomally by the action of a large multienzyme complex using a multicarrier thiotemplate mechanism. Recently, the operon responsible for its biosynthesis was cloned and subsequently sequenced *(13,14)*. Knowledge of the modular arrangement of the peptide synthetases involved in the surfactin and lichenysin biosynthesis makes the lipopeptides ideally suited to a combinatorial biosynthetic search for more active or more refined molecules *(15,16)*. Furthermore, by taking advantage of the substrate variability of some binding sites of the enzyme subunits *(17)*, a set of variants, exhibiting homologies in their peptide sequences at positions 2, 4, or 7, has been obtained by using the directed-biosynthesis strategy *(1)*. Considering the structural characteristics, surfactin that possesses an L-Glu residue at position 1 instead of L-Gln in lichenysin is a dianionic amphiphile, whose surface and cation-complexing properties have been well described. In fact, it was previously demonstrated that the divalent cation-complexing property of surfactin is owing to the presence of the two negative charges, i.e., the aspartyl and glutamyl residues that, facing each other in the three-dimensional topology, defined a well-suited binding site for Ca^{2+} *(18)*. Ionized surfactin binds Ca^{2+} or other divalent cations forming a stable surfactin-cation 1:1 complex *(19,20)*, and the binding is accompanied by a conformational change of the peptide cycle *(20)*. Moreover, because the surfactin-Ca^{2+} complex is a neutral compound, it is

more deeply incorporated into lipid bilayers *(20,21)*. Surfactin acts also as an ionophore transporting monovalent and divalent cations across an inorganic solvent barrier *(19)*. All these results have to be linked with the properties of surfactin. In fact, we know that the surfactant capacity and the membrane-disrupting effect of surfactin were greatly enhanced by the presence of Ca^{2+} in the medium *(22)*. Ca^{2+} is currently thought to stabilize the surfactin conformation and to function as an assembly template for the micellization process *(23)*.

Because of the decisive role of carboxyl groups in the cation-binding process, the lack of such a group in lichenysin may greatly modify its ability to form a complex with cation. In the following, we describe the Ca^{2+} binding by lichenysin and the consequences on surfactant and membrane properties. A systematic study of surfactin peptidic variants varying in the length and branching of hydrophobic side chains has already enabled us to approach some structure-activity relationships *(24)*. The results presented here provide information on the role of carboxyl groups and usefully complete the aforementioned study.

Materials and Methods

Chemicals

Purified lichenysin and surfactin were prepared by semipreparative reverse phase high-performance liquid chromatography as previously described *(11)*. All the assays were performed with lipopeptides having a majority (>90%) of C_{15} β-hydroxy fatty acid.

Cation Binding Measurements

Measurements were made using the previously described conductimetric method of Wallach and Hanss *(25)*. A thermostatically controlled conductimetric cell was filled with 4 mL of 5 m*M* Tris solution at pH 9.4 containing 0.1 or 0.3 m*M* lipopeptide. A constant volume (10 µL) of the cation solution ($CaCl_2$ or $MgCl_2$) was successively added to the cell content while the conductance was recorded. The values were compared with those obtained in absence of lipopeptide. For each cation concentration, it was possible to deduce the concentrations of bound and free cations. Conductances were measured with a CD-810 conductimeter (Solea-Tacussel). The results were analyzed using Scatchard plots *(19)*. In another series of experiments, increasing quantities of each lipopeptide (from 0.01 to 0.16 m*M*) were added to the 5 m*M* Tris solution containing 0.6 m*M* $CaCl_2$. Then, bound Ca^{2+} was plotted vs lipopeptide concentration.

Surface Tension Measurements

Surface tension was measured at 25°C with a Krüss tensiometer by the procedure of Du Noüy with platinum ring, in dependance of concentration *(22)*. Lipopeptides were dissolved in a 5 m*M* Tris unbuffered solution at

pH 9.4 in the absence or presence of 0.1 m*M* $CaCl_2$. Some assays were also performed in a 0.1 *M* $NaHCO_3$ solution at pH 9.0. The surface tension–concentration plots were used to determine critical micellar concentration (c.m.c.) and the surface tension close to c.m.c., γ_{cmc}. From the slope of the decreasing segment before the premicellar state, we can calculate Γ_{max}, the interfacial concentration, by application of the Gibbs adsorption equation, $\Gamma_{max} = 1/RT(dg/d \ln C)$, and the interfacial molecular area is $a = 1/\Gamma_{max}$.

Monolayer experiments were carried out by using a homemade Langmuir film balance. The Teflon trough rested on an antivibration plate and was enclosed in a Plexiglas box. Lipopeptide solutions in chloroform: methanol (1:1 [v/v]) were spread on the subphase (5 m*M* Tris, pH 9.4) with a 50-µL Hamilton syringe, and the solvent was allowed to evaporate. Lipopeptide monolayers were then compressed at a rate of 0.1 nm^2 (mol·min) by means of a step-by-step motor, and the compression isotherm curves were recorded. The compressibility coefficient of the monolayer $\beta_0 = -(1/A_0)(dA/dT)_T$ is calculated from the isotherm parameters *(20)*; A_0 is the intersection of the tangent to the expanded part of the isotherm with the area axis.

Hemolysis of Erythrocytes

Fresh human blood was supplied by voluntary donors and collected on 3.8% sodium citrate. The erythrocytes were sedimented by centrifugation at 4000*g* for 10 min and washed twice with 150 m*M* NaCl and once with a synthetic plasma described by Chen et al. *(26)* that contained no inorganic cations, a prerequisite for measurement of the effect of cations on hemolysis. The erythrocytes were then suspended in this plasma at a hematocrit of 1%. Various concentrations of lipopeptides, in the absence or presence of 0.1 m*M* $CaCl_2$, were added to the suspensions, then incubated at 37°C, and, at time intervals, aliquots were centrifuged. The release of hemoglobin was determined by measuring the absorbance of supernatants at 540 nm. Minimal hemolytic concentration (m.h.c.) was defined as inducing 100% hemolysis.

Results

Influence of Cations on Interfacial Parameters of Lichenysin

In the absence of cations, lichenysin was shown to be a more efficient surfactant than surfactin. Indeed, the c.m.c. of lichensyin was found to be 10-fold lower than that of surfactin, i.e., 22 µ*M* instead of 220 µ*M*, and the γ_{cmc} reached 35 mN/m instead of 37 mN/m. This result pointed out the predominant role of the polar domain in the aggregation process *(11)*. Here, we have studied the effect of cations on the interfacial parameters of both lipopeptides. As shown in Table 1, we could observe a decrease in both c.m.c. and γ_{cmc} values. For lichenysin, the c.m.c. was reduced from 22 to

Table 1
Interfacial Characteristics of Lichenysin and Surfactin, in Absence and Presence of Cations (Ca^{2+} and Na^{+})

	c.m.c. (μ*M*)			γ_{cmc} (mN/m)			Γ_{max} (mol/nm^2)	Molecular area (nm^2/mol)[a]	Compressibility coefficient β_0 (m/*N*)
	Tris (5 m*M*)	Tris (5 m*M*) + $CaCl_2$ (0.1 m*M*)	$NaHCO_3$ (0.1 *M*)	Tris (5 m*M*)	Tris (5 m*M*) + $CaCl_2$ (0.1 m*M*)	$NaHCO_3$ (0.1 *M*)	Tris (5 m*M*)	Tris (5 m*M*)	Tris (5 m*M*)
Lichenysin	22	12	17	35	32	30	2.56	0.39	15.5
Surfactin	220	20	9	37	28	29	1.18	0.85	10.5

[a]Calculated at concentrations below the premicellar state of each lipopeptide. Values are the mean of two experiments. γ_{cmc} values differ from less than ± 0.5 mN/m; c.m.c. values differ from less than ±20 μ*M* for surfactin and ±5 μ*M* for lichenysin.

12 μ*M* in the presence of Ca^{2+} and γ_{cmc} was lowered by 3 U. These changes are of marginal significance compared to surfactin, which was found to be more sensitive. In the same experimental conditions, the latter's c.m.c. was lowered from 220 to 20 μ*M* and the γ_{cmc} by 9 U. The monovalent cation Na^+ induced approximately the same effects but at a concentration 20-fold higher. In this context, the original disparity between the surfactant activities of both lipopeptides is practically abolished.

Comparison of Lichenysin and Surfactin Binding with Ca^{2+} and Mg^{2+}

Although the modulating effect of cations seemed to be globally the same on the surface properties of both lipopeptides, the presence of a unique carboxyl group in lichenysin vs two carboxyl groups in surfactin suggested a quite different mode of chelation with divalent cations. Affinity for cations was monitored by conductimetry *(19)*. Figure 1 reports the Scatchard's representation of the binding with Ca^{2+} (Fig. 1A) and Mg^{2+} (Fig. 1B). Table 2 summarizes the association constants (K_A) as well as the lipopeptide-cation molar ratios (*n*). Association constants of lichenysin with Ca^{2+} and Mg^{2+} are 4-fold and 10-fold higher, respectively, than those of surfactin. In addition, the curves agree with the formation of a lichenysin-cation complex at a molar ratio of 2:1 instead of 1:1 for surfactin. Thus, a second experiment was used to check the validity of these results. Figure 2 shows bound Ca^{2+} at different lipopeptide concentrations. For all the tested concentrations, the amount of Ca^{2+} bound by surfactin is double that bound by lichenysin, thus fully supporting the 2:1 ratio found for lichenysin.

From these results, it is obvious that the monoanionic lichenysin is a better chelating agent than surfactin, with a slight selectivity for Ca^{2+}. The 1:1 surfactin-Ca^{2+} ratio had suggested the formation of an intramolecular complex, corroborating the results from the molecular modeling showing, in the surfactin topology, the existence of a "claw" conformation created by the two acidic side chains at positions 1 and 5 *(18)*. Interestingly, the 2:1 molar ratio found for the lichenysin-Ca^{2+} complex strongly suggests the existence of intermolecular salt bridges and the formation of lichenysin dimers via divalent cations. The enhanced strength of the binding could be explained by a different reactivity of each carboxyl group toward cation. We might hypothesize that the intermolecular bridge between two β-COOH is more stable than the intramolecular bridge between one β-COOH and one γ-COOH in surfactin. In addition, the greater compactness of the molecule illustrated by the surface area value, i.e., 0.39 vs 0.85 nm^2 for surfactin, should favor the intermolecular interactions, with a consequently strengthened stability of the complex. Another favorable argument is provided by the comparison of the compression isotherms at the air/water interface (Fig. 3). Although the shape of the compression isotherms was found to be quite similar for both the molecules, the compressibility coefficient β_0 was increased about 35% for lichenysin (*see* Table 1), indicating a higher compressibility of the monolayer at the air/water interface. This result could be

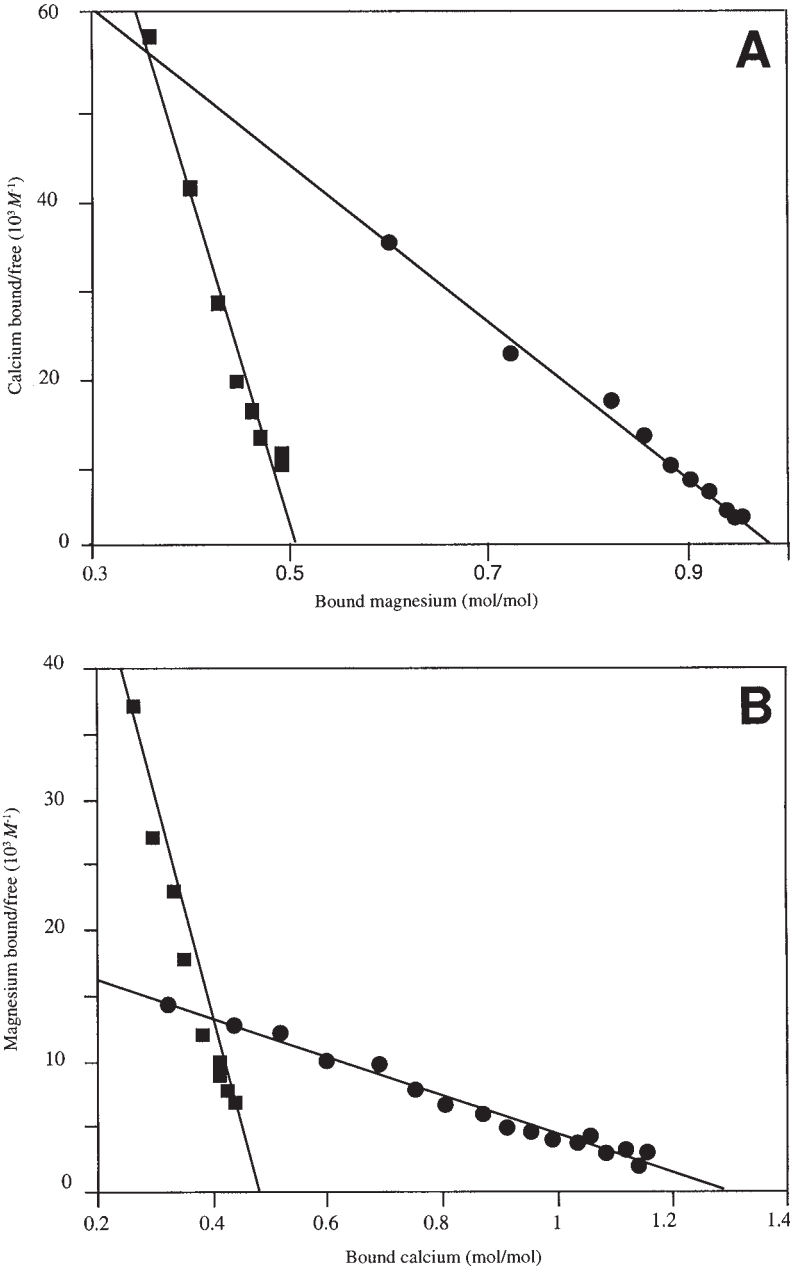

Fig. 1. Scatchard plots of Ca^{2+} binding **(A)** and Mg^{2+} binding **(B)** with 1×10^{-4} *M* lichenysin (■) and 3×10^{-4} *M* surfactin (●). The results were obtained by conductimetric method.

related to a weaker electrostatic repulsion with a progressive change in the orientation of the lichenysin peptide cycle that can adopt a more vertical position than surfactin *(20)*.

Table 2
Association Constants (K_A) and Molar Ratios of Lipopeptide/Cation (n) of Lichenysin Compared to Surfactin[a]

	Ca^{2+}		Mg^{2+}	
	K_A (10^5 M^{-1})	n	K_A (10^5 M^{-1})	n
Lichenysin	3.7 ± 0.30	0.51 ± 0.05	1.6 ± 0.30	0.47 ± 0.06
Surfactin	1.0 ± 0.11	0.98 ± 0.15	0.1 ± 0.02	1.25 ± 0.21

[a]Values are means of two or three experiments. n is the value at the origin of the vertical axis.

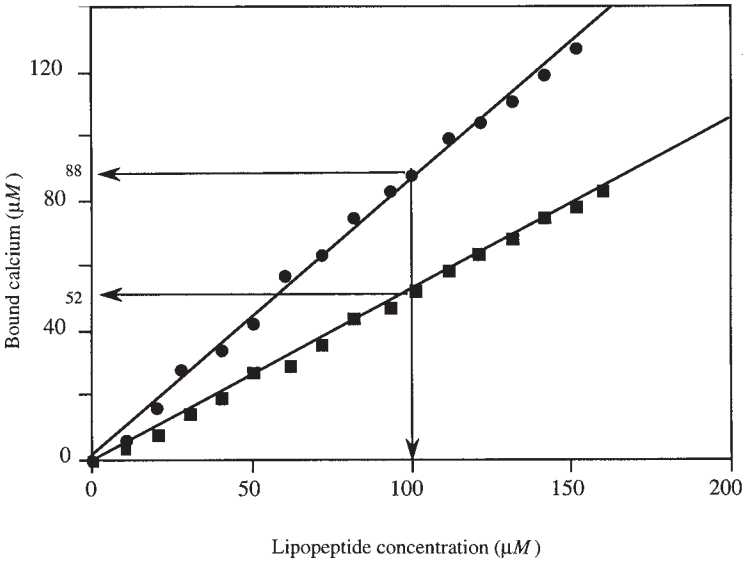

Fig. 2. Bound Ca^{2+} vs lipopeptide concentration: (●) surfactin; (■) lichenysin.

Activation by Ca^{2+} Ions of Lichenysin- and Surfactin-Induced Hemolysis

Biological consequences of the interactions with cations were tested on erythrocyte membranes. Figure 4 shows the hemolysis of human erythrocytes induced by lichenysin and surfactin in absence and presence of calcium. In the absence of cation, lichenysin was 15-fold more hemolytic than surfactin because the m.h.c. decreased from 200 to 15 μ*M*, a value quite close to that of the Glu-γ-methyl ester surfactin, a monoanionic variant obtained by selective esterification *(27)* (Table 3). By contrast, surfactin-induced hemolysis was found to be more sensitive to the presence of cations because the m.h.c. was reduced by fivefold vs twofold for lichenysin. We can see that this ratio is roughly the same as that mentioned for the shifts in the c.m.c.

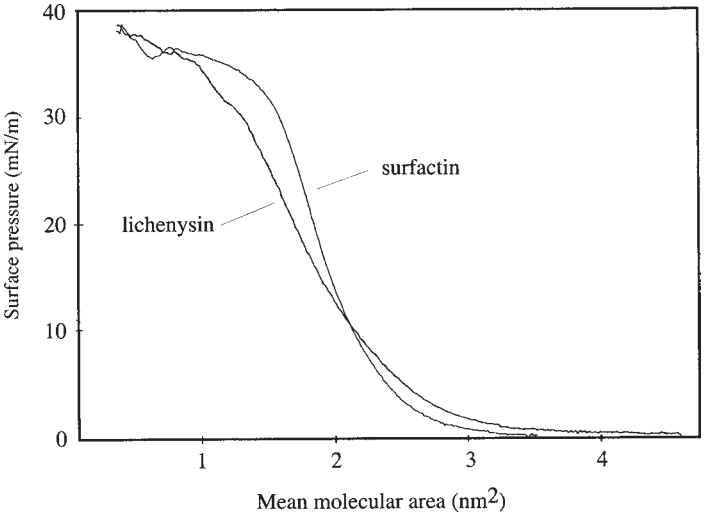

Fig. 3. Compression isotherms of lichenysin and surfactin at the air/water interface at 20°C (subphase: 5 m*M* Tris, pH 9.4).

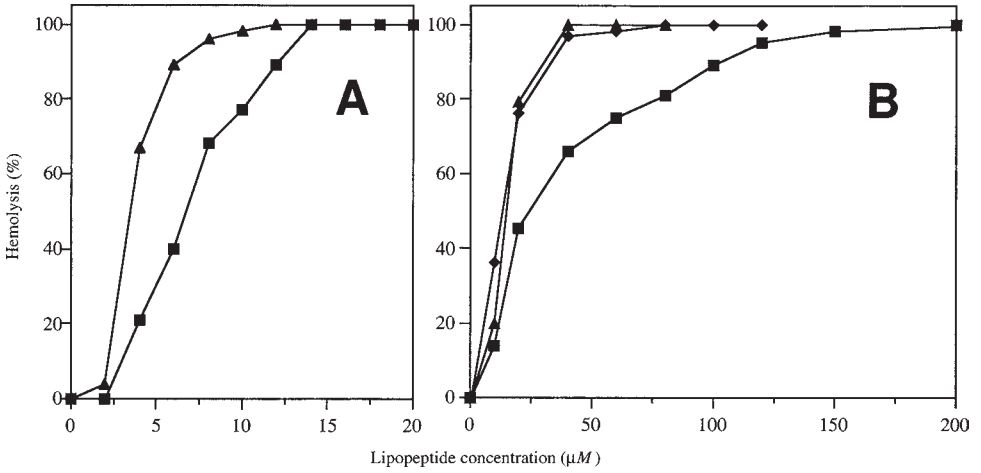

Fig. 4. Hemolysis of human erythrocytes at 37°C by lichenysin with (▲) and without Ca^{2+} (■) **(A)**, by surfactin with (▲) and without Ca^{2+} (■), and by the variant [Ile2,4,7]surfactin (◆) **(B)** (final hematocrit: 1%).

Table 3
Relationships Between c.m.c. and m.h.c. Values
of Lipopeptides of Surfactin Family

Lipopeptide	c.m.c. (μ*M*)	m.h.c. (μ*M*)
Surfactin	220	200
[Ile2,4,7]surfactin	90	60
Surfactin/Ca^{2+}	20	40
Surfactin Glu-γ-methyl ester	30	12
Lichenysin	22	15
Lichenysin/Ca^{2+}	12	8

The picture that emerges from this comparative study is that a major correlation can be made between the membrane disruptive effect of a lipopeptide and its self-association property. Interestingly, previous studies demonstrated that these two parameters were also modulated by the degree of hydrophobicity of some side chains in surfactin *(24)*. From these data, it appears that this factor has less influence than the negative charge of the carboxyl group. Table 3 illustrates that the c.m.c and m.h.c. values of the trisubstituted variant [Ile2,4,7]surfactin, i.e., (90 µ*M*) and (60 µ*M*), respectively, are intermediate between those of surfactin and lichenysin.

Discussion

Because substitutions of amino acids are frequently observed in surfactin sequence, numerous peptidic variants have been obtained that provided an important basis for the investigation of structure-activity relationships. Previous studies had already allowed researchers to distinguish finely the role of the different hydrophobic residues in the modulation of activities, according to their position in the peptide sequence *(24,28,29)*. With a view to clarifying the role of the carboxyl group, we have presented herein a comparative evaluation of the properties of surfactin with those of lichenysin, a naturally occurring variant differing from surfactin by one carboxyl group. Among the various activities of the molecule, surface, cation-binding, and hemolytic activities appeared as relevant parameters. Although the L-Glu/L-Gln exchange could be considered *a priori* as a minor change in the peptide moiety, it had important consequences on the properties of the molecule. In fact, lichenysin was a better surfactant as well as cation-chelating and hemolytic agent than surfactin: its c.m.c. and m.h.c. were reduced by 10- and 15-fold, respectively, as compared with surfactin. Then, the Ca^{2+} and Mg^{2+} binding constants were increased by a factor of 4 and 16, respectively. It is also interesting to note that lichenysin was less selective because the association constant with Ca^{2+} was only 2-fold higher than that with Mg^{2+} vs 10-fold higher for surfactin. The surfactin selectivity is believed to be essentially governed by the agreement between the steric hindrance of the cation and the size of the "claw" created by the two acidic side chains *(18)*. From this model, the disappearance of a negative charge suppresses this possibility. Consequently, the cation-binding site is less defined, and the lichenysin discrimination substantially decreases.

The cyclic backbone of surfactin displays a particular topology related to the sequence of L- and D-conformations. Because the chiral sequence is common to both lipopeptides, we may assume that the backbone architecture is not extensively modified by the Glu/Gln exchange. Therefore, the different binding property certainly correlates with a different side chain topology. More precisely, the orientation of the Gln side chain may be preferred for accessibility of the carboxyl group to cations. Such an effect has been previously demonstrated for a more conservative substitution in

the surfactin molecule *(24)*. Most remarkable is the lack of correlation between the selectivity and the strength of the binding, suggesting that other factors are involved in the stabilization of the cation. In fact, the lack of a negative charge also improves the propensity of the lipopeptide for intermolecular hydrophobic interactions, as illustrated by the drastic drop in the c.m.c. Favorable conjunction of these two factors, namely a change in the side chain topology and a more hydrophobic effect, contributes to the efficient stabilization of the cation.

Results from hemolytic activity measurements agree with a sharp correlation between the membrane-disruptive effect and the physical state of the lipopeptide in the solution. Such a relationship has been observed before with the natural variant [Ile2,4,7]surfactin (*see* Table 3). Moreover, its affinity for Ca^{2+} ions was increased over that of surfactin to an extent comparable to lichenysin. The higher binding of Ca^{2+}, which was ascribed to only the Leu-2 to Ile-2 substitution, was considered as a consequence of a local change in the steric hindrance of the side chain near the required carboxyl groups *(18,24)*. The results presented herein support this view, further validating the structure-activity relationships established from the three-dimensional model of surfactin. Regarding the effect of the cations on lichenysin activities, its marginal significance suggests that the hydrophobic effect owing to the reduction in the negative charge overrides the neutralizing effect promoted by the cations in the micellization process. However, in the objective of the template-assembled micelles approach described by Osman et al. *(23)*, evidence for the role of the cations is more relevant because they directly participate in the molecular assembly by promoting a dimer formation.

Lichenysin conformation is already under investigation, which should enable us to establish more precise conformation-activity relationships.

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