Nonribosomal peptide synthesis in Bacillus subtilis
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Chapter five
Effects of surfactin on lipid bilayers

Abstract

The nonribosomally synthesized lipopeptide surfactin, produced by various strains of Bacillus subtilis exhibits many activities. Here we present a study of the effects of surfactin on lipid bilayers. Surfactin induces partial leakage on entrapped carboxyfluorescein from vesicles, but hardly dissipates an induced ∆ψ, either inside positive or inside negative. Only at relatively high concentrations does surfactin induce a slow decrease of the induced ∆ψ. The efficiency of surfactin to induce carboxyfluorescein leakage is concentration dependent in a linear dose-response relationship. The degree of leakage was dependent on the pH, much stronger so at pH 6.0 than at pH 8.0. The hemolytic activity of surfactin was also pH dependent, also most strongly so at pH 6.0. Finally, induction of an inside positive ∆ψ increased the efficiency of surfactin-induced carboxyfluorescein leakage. However, an inside negative ∆ψ did not significantly influence the surfactin-induced carboxyfluorescein leakage. The results are interpreted on the basis of a hypothesis in which it is assumed that surfactin renders the membrane permeable allowing endogenous surfactin to diffuse to the external medium.

Introduction

The Gram-positive bacterium Bacillus subtilis is a well-known producer of nonribosomally synthesized peptides such as fengycin, surfactin and members of the iturin family, which contain a fatty acid moiety. Most of the lipopeptides are endowed with strong antifungal and hemolytic activities, but their antibacterial activity is usually limited (Arima et al., 1968; Vanittanakom et al., 1986; Marget-Dana and Peypoux, 1994). These lipopeptides are synthesized by large multienzyme complexes, peptide synthetases, which exhibit a modular structure. The modules are the minimal, independent units that catalyze all necessary reactions to modify and incorporate one specific amino acid into the growing peptide chain. The genetic arrangement of these modules is usually colinear with the amino acid sequence of the peptide product (Marahiel et al., 1997). Due to the easy genetic accessibility of B. subtilis much is known about the way these lipopeptides are synthesized. The elucidation of the primary structure revealed that surfactin is a macrolide lipopeptide containing the heptapeptide sequence L-Glu - L-Leu - D-Leu - L-Val - L-Asp - D-Leu - L-Leu linked to a β-hydroxy fatty acid with 13,14 or 15 carbon atoms (Fig. 5.1; Kakinuma et al., 1969a; Kakinuma et al., 1969b). Due to lack of specificity of the corresponding adenylation domains in the surfactin synthetase, variations can occur at specific positions in the peptide sequence (Grangemard et al., 1997). NMR techniques established that in solution and at the air/water interface surfactin exhibits a characteristic "horse saddle" conformation.
that presumably accounts for its broad spectrum of biological activities (Bonmatin et al., 1992; Bonmatin et al., 1994).

As might be expected from its amphiphilic nature, due to the polar amino acid head and the nonpolar hydrocarbon chain, surfactin is a powerful biosurfactant. It possesses a detergent-like action, lowers the surface tension of water from 72 mN m\(^{-1}\) to 27 mN m\(^{-1}\) at a concentration of 20 \(\mu\)M, and exhibits a strong tendency towards micellisation as well as foam formation (Ishigami et al., 1995; Razafindralambo et al., 1998; Peypoux et al., 1999). In addition to lowering surface tension, surfactin exhibits a wide range of other properties. It forms a stable 1/1 complex with divalent cations, penetrates phospholipid monolayers and induces temporary cation-selective leakage in membranes (Sheppard et al., 1991; Thimon et al., 1993; Maget-Dana and Ptak, 1995). Surfactin also inhibits several enzymes such as phospholipase A2 and various metal-ion dependent cAMP-phosphodiesterases as well as fibrin clot formation and tumor growth (Arima et al., 1968; Kameda et al., 1972; Kim et al., 1998). In contrast to the other lipopeptides produced by \(B.\ subtilis\), surfactin does not exhibit antifungal activity, but in addition to hemolytic activity also shows antiviral and antimycoplasma activities (Bernheimer and Avigad, 1970; Nissen et al., 1997; Vollenbroich et al., 1997). Finally, it has been shown that surfactin can be potentially used as a hypocholesterolemic agent (Imai et al., 1971). In contrast to the wide range of observed activities, the natural function of surfactin remains obscure, but it might function as a siderophore. In addition, it has been observed that surfactin influences the adhesiveness and hydrophobicity of \(B.\ subtilis\), and interacts with other lipopeptides produced by \(B.\ subtilis\) resulting in synergistic effects (Ahimou et al., 2000; Maget-dana et al., 1992; Thimon et al., 1992).

Since attempts to identify proteins involved in the secretion of surfactin failed, and, so far, no protein has been reported unequivocally involved in secretion of lipopeptides, it can not be excluded that the secretion of surfactin is a passive process. However, such a model is only conceivable if surfactin does not perturb the integrity of the cytoplasmic membrane in a gross way. Accordingly, this study was undertaken to determine the capacity of surfactin to permeabilise membranes composed of the zwitterionic phosphatidylcholine and to examine whether it would allow the passage of the rather large carboxyfluorescein molecule.

**Materials and methods**

**Chemicals.** Bovine heart phosphatidylcholine (PC) lipids used in this study for preparation of vesicles, like the fluorescent probes 5(6)-carboxyfluorescein (CF) and 3,3’-dipropyl-thiadicarbocyanine iodide (DISC3(5)) used for efflux and \(\Delta\psi\) measurements, respectively, were obtained from Sigma (St. Louis, USA). All other chemicals used were obtained from Sigma (St. Louis, USA) and Merck KGaA (Darmstadt, Germany).

**Preparation of large unilamellar phospholipid vesicles.** Large unilamellar PC vesicles with a diameter of 200 nm were prepared by the extrusion method and treated as previously described (Hope et al., 1985; Breukink et al., 1997), and diluted to a final concentration of 15 mg/ml.
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phospholipid. 5(6)-carboxyfluorescein (CF)-loaded vesicles were prepared in the presence of 50mM CF, and the extravesicular CF was removed by gel filtration using a Sephadex G-25 column (Amersham Pharmacia Biotech, Little Chalfont, UK), after which the vesicles were diluted to a final concentration of 15 mg/ml phospholipid. Dependent on the experiment vesicles were prepared in 50 mM potassium-phosphate or sodium phosphate buffers with various pH values. All fluorescence measurements were performed using the luminescence spectrometer LS 50B (Perkin Elmer, Shelton, USA).

**CF efflux assay.** CF efflux assays were performed essentially using the method described earlier (Weinstein et al., 1980). Dependent on purpose surfactin-induced CF leakage was determined in various 50 mM potassium-phosphate or sodium phosphate buffers. For each surfactin-induced CF leakage assay 10 µl CF loaded vesicles was added to 1.8 ml buffer under continuous stirring and kept at 25°C for 1 minute, until a stable signal was obtained. After addition of various concentrations of surfactin, the surfactin-induced leakage was monitored for 3 minutes at several pH values, and in the presence of a ΔpH. The fluorescence increase due to decrease of self-quenching was monitored at 515 nm (excitation at 492 nm) and surfactin-induced CF leakage was expressed in absolute values or relative to the total amount of CF released after total disruption of the vesicles by the addition of 10 µl of 20% Triton X-100.

**Membrane potential measurements.** The generation and dissipation of a transmembrane electrical potential, Δψ, was monitored using the fluorescent probe DISC3(5) as previously described (Sims et al., 1974). A Δψ, either inside positive or inside negative, was generated using the potassium-ion (K⁺) specific ionophore valinomycin. For generation of an inside negative membrane potential (Δψ), vesicles containing 50 mM potassium-phosphate buffer were diluted in a 50 mM sodium-phosphate buffer after which valinomycin was added, whereas for the generation of an inside positive Δψ, vesicles containing 50 mM sodium-phosphate buffer were diluted in a 50 mM potassium-phosphate buffer after which valinomycin was added. The effect of surfactin on Δψ was monitored by diluting 5 µl vesicles in 1.8 ml buffer after which DISC3(5) was added to a final concentration of 1.5 µM. Subsequently, valinomycin was added to a final concentration of 125 nM and the mixture was kept at 25°C for 1 minute under continuous stirring until a stable Δψ was obtained. After addition of various concentrations of surfactin, the surfactin-induced dissipation of the Δψ was monitored for about 5 minutes, after which the Δψ was completely dissipated by addition of 1µl of 0.5 mM nigericine, a potassium ion/proton exchanger. The fluorescence was monitored at 666 nM (excitation at 643 nM).

**Hemolytic activity measurements.** The hemolytic activity of surfactin was determined in 500-fold diluted sheep blood in potassium phosphate or sodium phosphate buffers with various pH values. Surfactin was added, and the induced lysis of blood cells was determined with a photospectrometer lambda 11 (Perkin Elmer, Shelton, USA) at 600 nM.

**Results**

**Surfactin induces carboxyfluorescein leakage.** To study the effects of surfactin on the permeability of lipid bilayers, various concentration of surfactin were added to CF-loaded phosphatidylcholine vesicles, and the capability of surfactin to induce CF leakage was determined. To determine the
maximum fluorescence, corresponding with 100% leakage, 0.2% of triton X-100 was added to totally dissolve the loaded vesicles. Addition of 60 µM of surfactin resulted in almost complete leakage of CF from the vesicles in 10 minutes at pH 7.0, clearly demonstrating the capability of surfactin to render the vesicle membranes permeable for CF (Fig. 5.2A).

To examine whether surfactin-induced CF leakage was concentration dependent, increasing concentrations of surfactin were added to a constant amount of CF-loaded vesicles, and the induced leakage was determined 4.0 minutes after addition and expressed relatively to the maximal leakage caused by total disruption of the loaded vesicles. These measurements revealed that the surfactin-induced CF leakage was concentration dependent in a linear dose-response fashion (Fig. 5.2B).

**Surfactin-induced carboxyfluorescein leakage is pH dependent.** Dependent on the pH, the peptide moiety of surfactin contains two negatively charged amino acids, aspartate and glutamate. To study the role of these charged amino acid residues and the influence of the pH on the effects of surfactin on lipid bilayers, surfactin-induced CF leakage was determined at pH 6.0, and pH 8.0. At both pH values surfactin causes CF leakage (Fig. 5.3A and B). However, the efficiency of surfactin in inducing CF-leakage from vesicles is much more pronounced at the lower pH.

As the fluorescence of CF is pH dependent, we corrected for this effect by expressing the induced CF leakage from vesicles 4.0 minutes after addition of 20 µM surfactin at various pH values relative to the maximal fluorescence (Fig. 5.3C). Clearly the surfactin-induced CF leakage is pH dependent and increases at lower pH values. Upon addition of increasing amounts of surfactin at pH 6.0 and pH 8.0 the induced CF leakage increased also at these pH values in a linear dose-response fashion (Fig. 5.3D). Similar results were
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Figure 5.3. Effect of pH on surfactin induced CF leakage. A) Leakage induced by addition of 40 µM surfactin. at pH 6.0 (A), and at pH 8.0 (B). Arrows indicate time points of addition of surfactin (1) and Triton X-100 (2). C) Induced leakage after addition of 20 µM of surfactin relatively to the maximal leakage after addition of Triton X-100 at various pH values. D) Induced leakage, relatively to the maximal leakage after addition of Triton X-100, 4 minutes after addition of various concentration of surfactin at pH 6.0 (▲) and pH 8.0 (●).

obtained for the hemolytic activity of surfactin (data not shown).

**Surfactin hardly causes dissipation of the membrane potential** Δψ. It has been described that surfactin induces selective cationic channels in lipid bilayers in the presence, which, at higher salt concentrations, involves a dimer (Sheppard et al. 1991). These results suggest that surfactin might dissipate a Δψ. To examine this possibility a Δψ, either inside positive or inside negative, was generated and the effects of surfactin on Δψ were monitored using DISC3(5). Upon addition of various concentrations of surfactin, an immediate small transient dissipation of the Δψ was
observed, which had disappeared already after 20 seconds (Fig. 5.4A). Only when the concentration of surfactin was raised to 40 µM a slow, continuing dissipation of the Δψ was observed after the initial transient dissipation (Fig. 5.4B). Essentially similar results were obtained at pH 6.0 and pH 8.0, irrespective of whether the Δψ was inside negative or inside positive (data not shown). These results demonstrate that surfactin hardly affects the permeability of the membranes for small ions and does not perturb the structural integrity of the vesicles in a gross way.

**Effect of Δψ on surfactin-induced carboxyfluorescein leakage.** To study whether a Δψ influences the efficiency of surfactin to induce CF leakage from loaded vesicles, a Δψ was generated and after addition of various concentrations of surfactin CF leakage was compared with that in the absence of a Δψ at pH 6.0, pH 7.0 and pH 8.0. First the surfactin-induced CF leakage was compared in the presence of K⁺ or Na⁺ ions, and in the absence and presence of valinomycin without generating a Δψ. No differences were observed indicating that surfactin-induced CF leakage is not affected by the presence of different ions and valinomycin (data not shown). In the presence of an inside positive Δψ, a strong stimulating effect was observed on surfactin-induced CF leakage at pH 7.0 (Fig. 5.5B). At pH 6.0 and pH 8.0 only a brief stimulating effect on the initial rate of leakage immediately after addition of surfactin was observed although after 3 minutes the total leakage was comparable (Fig. 5.5A and 5.5C). To minimize the possibility that the effects at pH 7.0 were due to osmotic effects, these experiment were repeated with additional 50 mM potassium phosphate added to the external buffer. The results were similar: again a strong stimulating effect was observed (Fig. 5.5D). In contrast, no effect on surfactin-induced CF leakage could be observed when an inside negative Δψ was generated (data not shown).
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Discussion

Earlier studies on the effects of surfactin in lipid monolayers as well as lipid bilayers revealed that surfactin interacts with phosphatidylcholine and phosphatidylglycerol, and that the insertion of the ionized form of surfactin into negatively charged bilayers is stimulated by Ca^{2+} ions. It appeared that surfactin penetrates spontaneously into lipid membranes by means of hydrophobic interactions (Deleu et al., 1999). In the present work we studied the effects of surfactin on lipid bilayers, using fluorescent probes. This was done by determining the efficiency of surfactin to induce leakage of CF entrapped in
phosphatidylcholine vesicles. DISC$_3$(5) was used to monitor the effect of surfactin on a generated $\Delta\psi$.

The addition of surfactin clearly induced CF leakage, which demonstrates the capability of surfactin to increase the permeability of phosphatidylcholine bilayers for reasonably large molecules. The induction of CF leakage by surfactin was found to be linearly dependent on the concentration of the lipopeptide. However, the efficiency of surfactin to induce CF leakage was rather low: a concentration as high as 40 $\mu$M of surfactin per 10 $\mu$M lipids was needed to effect almost complete CF leakage. Surfactin-induced CF leakage proved to be pH dependent, being most effective at pH 6.0, less effective at pH 7.0 and relatively ineffective at pH 8.0. At all three pH values, the induced leakage was linearly dependent on the concentration of surfactin. Similar results were obtained for the hemolytic activity of surfactin, which was most pronounced at lower pH values (data not shown). This pH dependence suggests that CF leakage and hemolysis is mainly induced by the partially or completely protonated form of surfactin, which might be in accordance with earlier studies showing that addition of Ca$^{2+}$, neutralizing the negative charges of surfactin, resulted in a deeper insertion into lipid membranes, possibly resulting from conformational changes of the molecule (Maget-Dana and Ptak, 1995).

The capability of surfactin to cause dissipation of a membrane potential, $\Delta\psi$, at pH 6.0, pH 7.0 and pH 8.0 was very limited under the conditions used, and no differences were observed between an inside positive $\Delta\psi$ and an inside negative $\Delta\psi$. Upon addition of surfactin a small, transient dissipation of the $\Delta\psi$ was observed and only addition of relatively high concentrations (40 $\mu$M) of surfactin resulted in a subsequent slow dissipation of the $\Delta\psi$. These results are in accordance with earlier studies, which showed that addition of surfactin results in brief conductance jumps due to transient formation of cation-specific pores (Sheppard et al., 1991).

Surfactin-induced CF leakage appeared to be influenced by the $\Delta\psi$. When an inside positive $\Delta\psi$ was generated, the surfactin-induced CF leakage at pH 7.0 was clearly stimulated. This effect was not seen in the presence of an inside negative $\Delta\psi$. The stimulating effect on the surfactin-induced CF leakage of an inside positive $\Delta\psi$ at pH 6.0 and pH 8.0 was marginal: only the initial rate of leakage seemed to be some what increased and the total leakage after 3 minutes was comparable. Finally, preliminary measurements seem to suggest that surfactin-induced CF leakage was independent of a $\Delta$pH (data not shown).

Taken together the present data show that surfactin hardly affects the stability of lipid bilayers because the surfactin-induced permeability for small ions remained very low, but increases the permeability for relatively large molecules. We might speculate that surfactin could facilitate its own translocation over the cytoplasmic membrane comparable to the CF-efflux. Such spontaneous translocation has also been reported for the basic amphiphilic peptide melittin (Matzuki et al., 1997). It is noteworthy that this spontaneous translocation is stimulated by the presence of an outside positive $\Delta\psi$, a situation prevailing in living cells. Spontaneous translocation would also explain why all attempts to identify proteins facilitating secretion of surfactin or other lipopeptides have, so far, failed. Although recently a membrane protein was identified in $B$. subtilis, homologous to proton motive force-dependent efflux pumps, which seemed to be involved in surfactin secretion and self-resistance towards surfactin, deletion of the encoding gene only decreased surfactin
accumulation in the culture medium. However, whether the decreased accumulation observed did not result from decreased expression of the srfA operon, was not determined (Tsuge et al., 2001).

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