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Iturin lipopeptides: interactions of mycosubtilin with lipids in planar membranes and mixed monolayers

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The interactions between the antifungal lipopeptide mycosubtilin and lipids are studied. Mycosubtilin increases the ion permeability of planar lipid membranes by forming ion conducting pores. The lifetime of these pores is greatly increased when the membrane contains cholesterol. In mixed monolayers the interaction between mycosubtilin and DMPC leads to the formation of a mycosubtilin/DMPC 1:2 complex non miscible in the excess DMPC monolayer but miscible in the mycosubtilin monolayer. Mycosubtilin and cholesterol interact strongly in monolayers in all proportions and form a mycosubtilin-cholesterol (1:2) complex. These results are analyzed with reference to the overall view of the activity of iturins and the importance of the lipopeptide conformation is outlined.

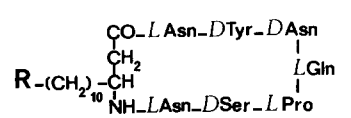
Introduction

Mycosubtilin belongs to the iturin family of lipopeptides produced by strains of *Bacillus subtilis* and which exhibit an antifungal activity [1] related to their interaction with the cytoplasmic membrane of target cells [2]. This activity closely depends on the structure of the cyclic peptide moiety (Fig. 1) as illustrated by the methylation of the hydroxyl group of the invariable D-Tyr-2 residue which considerably reduces the effects of iturins [3] or by the sequence inversion of the two adjacent D-Ser-6-L-Asn-7 residues [4] which makes mycosubtilin more active than iturin A [1]. It has been shown that this sequence inversion, which is a minor local variation, leads to quite different conformations in solution [5,6]. In this paper we report the specific properties of mycosubtilin with regard to its capacity to modify the ionic permeability of membranes and to interact with lipids in monolayers. The aim of this work is to investigate the incidence of a conformation change on the behaviour of iturin lipopeptides in lipid membranes.

Materials and Methods

Mycosubtilin was prepared in the Laboratoire de Biochimie Microbienne (Lyon) according to Ref. 7. Diphytanoylphosphatidylcholine was from Avanti, dimyristoylphosphatidylcholine from Calbiochem; cholesterol from Prolabo was recrystallized three times from methanol before use. Pyridine, hexafluoroisopropanol, hexane and absolute ethanol were from Merck. Cyclohexane was from Fluka. Pure water was obtained from a Millipore (Milli Q) apparatus.

Planar lipid membranes were formed from lipid vesicle suspension [8] on a hole (0.2 mm diameter) in a Parafilm® septum separating two identical KCl solutions and details of the apparatus have been described previously [9]. The electrical measurements were made via a pair of Ag/AgCl electrodes. The determination of ion selectivity was done from the measurement of the 'zero-current potential', V_Z, when a concentration



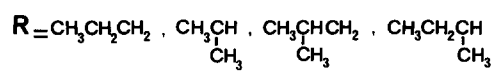


Fig. 1. Primary structure of mycosubtilin.

Abbreviation: DMPC, dimyristoylphosphatidylcholine.

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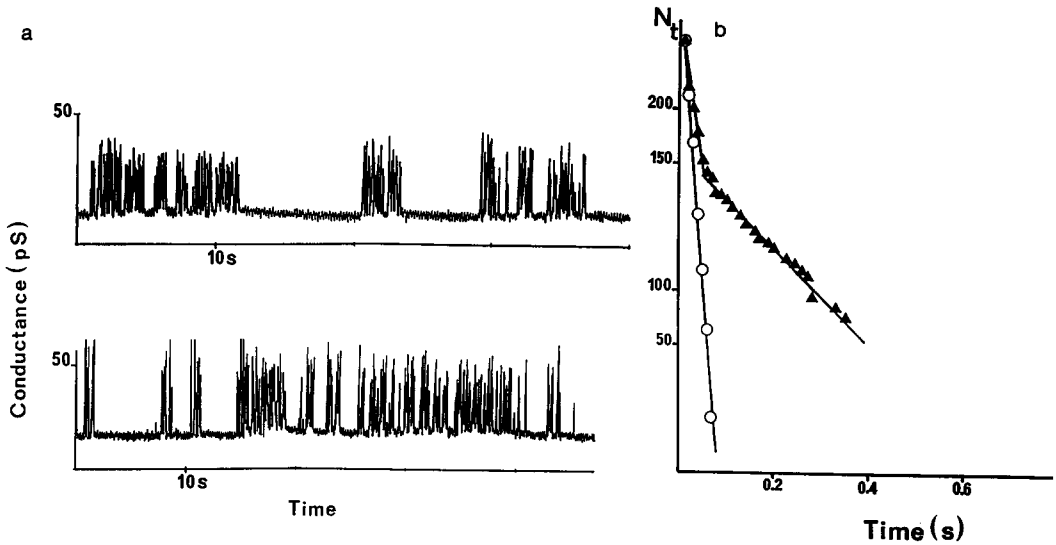

Fig. 2. Membrane current fluctuations induced by mycosubtilin. The planar membrane was made from diphytanoylphosphatidylcholine vesicles in 1 M KCl. (a) Current traces obtained about 10 min after the beginning of two separate experiments. (b) Distribution of the dwell-times in open (○) and closed (▲) state. The interburst dwell-time distribution is not shown. N_t is the number of individual dwell-times longer than t. The data are from the first trace. The plot is semilogarithmic.

gradient of KCl was established across the membrane. The membrane potential, V_m, was then deduced from the relation: $V_Z = V_m + (RT/F)\ \ln(a_1/a_2)$ where a is the KCl activity on each side 1 and 2 of the membrane.

Monolayer experiments were done using a Langmuir film balance system previously described [10]. Mycosubtilin was first dissolved in a minimum volume of pure pyridine and the solution diluted in the following solvent mixture: pyridine/cyclohexane/ethanol/hexafluoroisopropanol/hexane (56 : 13 : 13 : 10 : 8, by vol.). Lipids were dissolved in this solvent mixture. The mycosubtilin and lipid solutions were mixed before spreading at the air/water interface of pure distilled water with a 50-μl Hamilton microsyringe. The isotherm curves were recorded by compression of the film at a rate comprised between 0.02 and 0.05 $nm^2 \cdot mol^{-1} \cdot min^{-1}$. The temperature in the trough was maintained at 20 °C by circulating water and controlled with a thermocouple ($\Delta T = \pm 0.2$ °C).

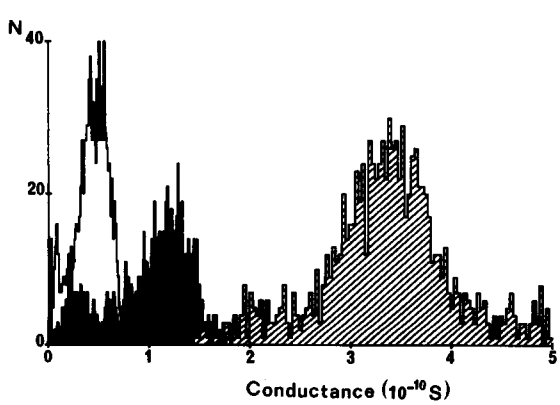

Fig. 3. Evolution with time of the conductance of the pores induced by mycosubtilin in diphytanoyl-PC membranes. Conductance histograms obtained 12 min, (□), 45 min (■) and 100 min (▨) after the formation of the membrane. N is the number of events.

Results

Permeability induced by mycosubtilin in planar lipid bilayers

Fig. 2 represents records of the membrane current fluctuations induced by mycosubtilin in a pure

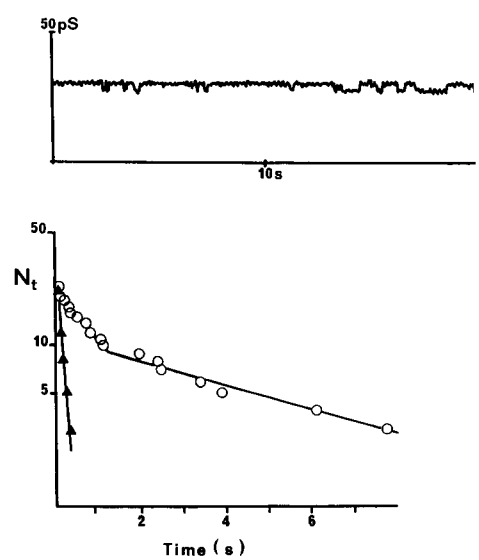

Fig. 4. Influence of cholesterol on the characteristics of the pores induced by mycosubtilin. The planar membrane was made from diphytanoyl-PC/cholesterol (2 : 1) vesicles in 1 M KCl. (a) Current trace. 90 min after the formation of the membrane. (b) Distribution of the dwell-times in open (○) and closed (▲) state. N_t is the number of individual dwell-times longer than t. The plot is semi-logarithmic.

phosphatidylcholine bilayer. The current flickers very quickly and the trace has the appearance of 'bursts'. The current jumps observed are due to the formation of ionic pores fluctuating between two states: the open and the closed one. We have studied the kinetics of opening and closing of the pores. The mean dwell time in the open state is very short (31 ms). The semi-log plot giving the distribution of the dwell time in closed state is biphasic showing the existence of two mean times (53 ms and 322 ms). The resting state between two bursting periods may last more than 10 s.

The conductance $G = I/V$ of these fluctuations is not dependent on the potential applied. Its values increases with time as shown Fig. 3.

The characteristics of the ionic pores induced by mycosubtilin change when the membrane contains cholesterol. First the unit conductance is less than 10 pS, 90 min after the beginning of the experiment (Fig. 4). Furthermore the dwell time in the open state is very much longer than in the case of pure phosphatidylcholine: we observe two mean times of 1.3 s and 6 s. On the other hand, the ionic pores do not stay a long time in the closed state. The mean dwell time is then 140 ms.

The ion selectivity induced in the membrane by mycosubtilin is given by the permeability ratio P_K/P_{Cl} and is calculated from the value of the membrane potential V_m when there are two different KCl concentrations on each side of the membrane.

According to the Goldman-Hodgkin-Katz equation [11]

$$V_m = RT/F \ln \frac{a_2 P_K/P_{Cl} + a_1}{a_1 P_K/P_{Cl} + a_2}$$

(where a_1 and a_2 are the KCl activity on side 1 and 2 of the membrane).

When the activity ratio $a_1/a_2 = 10.85$, $V_m = 13.8$ mV (data not shown) from which $P_K/P_{Cl} \approx 0.5$. Then, when a membrane is doped with mycosubtilin it becomes slightly anion selective.

Monolayer experiments

The isoherm (π–A) curve of pure mycosubtilin, is shown Fig. 5. It presents an inflection at 26 mN · m^{-1}, called transition pressure and corresponding to a rearrangement of the mycosubtilin molecules at the interface. We say that they pass from an expanded state (i) to a more condensed state (j). Then the surface pressure increases more slowly until a break point at 43 mN · m^{-1}. This point is called 'collapse pressure' although the pressure continues to increase upon further compression. The mean molecular area, A, is around 1 nm^2 at the beginning of the compression and around 0.35 nm^2 at the collapse pressure.

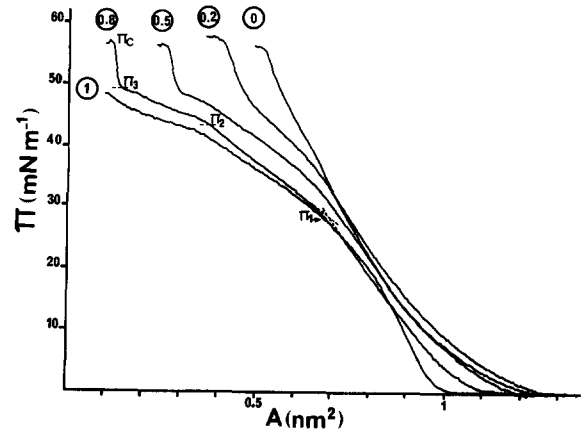

Fig. 5. Isotherm curves of mixed mycosubtilin/DMPC monolayers. The molar fraction x_M is indicated at the top of the curves. Pure DMPC (0); pure mycosubtilin (1).

Mycosubtilin / DMPC mixed monolayers

Isotherm (π–A) curves of mixed monolayers of mycosubtilin and DMPC have been plotted for different mycosubtilin molar fractions $x_M = n_M/(n_M + n_L)$, (n: number of molecules at the interface; the subscripts M and L refer, respectively, to mycosubtilin and lipid). Some of these curves are shown in Fig. 5.

The isotherm curve of a mixed monolayer presents a first transition pressure π_1, two break points at π_2 and π_3 assumed as two other pressure transitions and a collapse pressure π_c. These different transition pressures appear clearly on the isotherm corresponding to $x_M = 0.8$.

The composition dependence of the transition pressures is shown Fig. 6.

π_1 has a constant value of 37 mN · m^{-1} from $x_M = 0.02$ to 0.35. Then, at higher molar fractions of mycosubtilin, π_1 decreases continuously until the transition pressure of pure mycosubtilin monolayer ($x_M = 1$).

This result suggests the formation of a mycosubtilin-DMPC complex with a limiting stoichiometry, corresponding to $x_M = 0.35$. If we apply the phase rule [12] simplified in this case (at constant temperature and external pressure) as $F = 3 - \phi$ (with F, number of freedom degrees, and ϕ, number of surface phases in equilibrium with each other) we can predict that along the line ab, there are three phases ($F = 0$) in equilibrium with each other that should be: DMPC monolayer, mycosubtilin-DMPC complex monolayer in the expanded state (i) and complex monolayer in the more condensed state (j). Then in the region IA, the phase is not homogeneous and the mycosubtilin-DMPC complex is not miscible with the excess DMPC in the monolayer. The transition pressure of the complex is 37 mN · m^{-1} (point b). The mycosubtilin-DMPC complex is miscible in the 'excess' mycosubtilin monolayer ($F = 1$), and along the line bc, π_1 data are well described by the relation $\pi_1 = 25.6 - 11.5 \ln x_M$ (correlation coeffi-

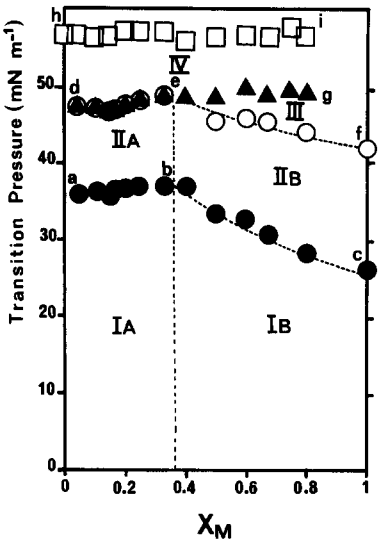

Fig. 6. Transition pressure-composition phase diagram for mycosubtilin/DMPC monolayers. π_1 (●), π_2 (○), π_3 (▲) and π_c (□) (as defined in the text and Fig. 4). The dotted line bc is drawn according to the relation $\pi_1 = 25.6 - 11.5 \cdot \ln x_M$. The dotted line ef is drawn according to $\pi_2 = 42.3 - 6.7 \cdot \ln\ x_M$. IA region: DMPC monolayer in the expanded state and complex monolayer in the expanded state (not miscible). IB region: homogeneous mixture of complex and mycosubtilin monolayer in the expanded state. IIA region: DMPC monolayer in the expanded state and complex monolayer in a condensed state (not miscible). IIB region: homogeneous mixture of complex and mycosubtilin monolayers in a condensed state. III region: complex monolayer in the condensed state and mycosubtilin in a bulk state (not miscible). IV region: DMPC monolayer in the expanded state and mycosubtilin in a bulk state (not miscible).

cient $r = 0.96$) expressed in $mN \cdot m^{-1}$. For an ideal miscibility the slope of the (π_1 – ln x_M) plot should be $kT/A_1 = 5.5\ mN \cdot m^{-1}$ (where $A_1 = 74 \cdot 10^{-20}\ m^2$ is the molecular area of mycosubtilin at π_1).

At low x_M, π_2 and π_3 are identical and their common value can be considered as constant (49 $mN \cdot m^{-1}$). Then π_3 stays at the same value (assumed to be the collapse pressure of the mycosubtilin/DMPC complex) while π_2 decreases continuously from 49 to 42 $mN \cdot m^{-1}$ (collapse pressure of pure mycosubtilin monolayer).

π_2 data are well described by the relation $\pi_2 = 42.3 - 6.7$ ln x_M (expressed in $mN \cdot m^{-1}$) ($r = 0.946$).

For an ideal miscilibility the slope of the (π_2 – ln x_M) plot should be $kT/A_2 = 12.25\ mN \cdot m^{-1}$ (where $A_2 = 33 \cdot 10^{-20}\ m^2$ is the molecular area of pure mycosubtilin at π_2).

The application of the phase rule allows us to predict that along the line ef two phases ($F = 1$) are in equilibrium with each other: an homogeneous phase of mycosubtilin-DMPC complex and 'excess' mycosubtilin and a bulk phase of mycosubtilin.

The collapse pressure of the mixed monolayers is always the same than the collapse pressure of pure DMPC monolayer (57 $mN \cdot m^{-1}$). Along the line hi, $F = 0$, then three phases are in equilibrium: DMPC monolayer, mycosubtilin in a bulk phase and DMPC in a bulk phase. This analysis is confirmed by the examination of the apparent molecular area of the mixed monolayers at the collapse pressure π_c: these areas correspond to the partial molecular areas of DMPC (to within 6%).

In Fig. 7 are plotted the values of the mean molecular area of the mixed monolayers as a function of composition, at a given pressure (data taken from the isotherms). These plots show positive deviations from the additivity rule [13] whatever the pressure. We can distinguish three parts in the (A – x_M) plots: part I, from $x_M = 0.02$ to 0.1, range composition where the Raoults' law still applied, the positive deviation has the maximum amplitude. The discontinuity between part II and part III occurs near $x_M = 0.35$ and corresponds to the mycosubtilin-DMPC complex seen above. The apparent partial molecular area of mycosubtilin in the complex in the presence of an 'excess' of DMPC (part II) is very expanded (for more than 30%). In part III data are distributed along a straight line ($0.830 < r < 0.986$ depending on the considered pressure), and the partial molecular area of mycosubtilin is that of the pure mycosubtilin monolayer.

Mycosubtilin / cholesterol mixed monolayers

Fig. 8 shows some of the isotherm curves of mycosubtilin/cholesterol mixed monolayers of various compositions. When the amount of mycosubtilin in the monolayer is higher than $x_M = 0.25$, the isotherm curves present an inflection at $\pi_1 > 20\ mN \cdot m^{-1}$ and two break points at π_2 and π_3.

When the amount of mycosubtilin in the monolayer is low, the isotherm curves present a shoulder at a

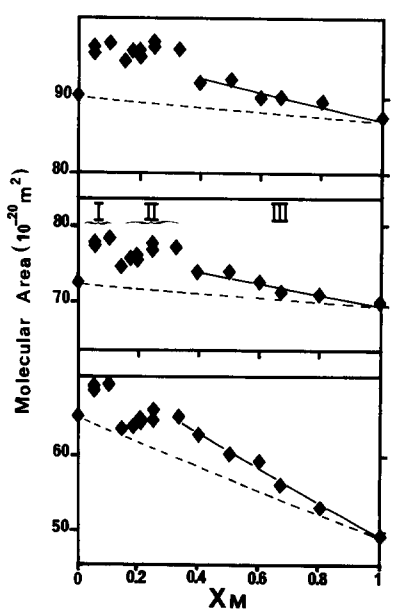

Fig. 7. Mean molecular areas of mixed mycosubtilin/DMPC monolayers as a function of composition at various surface pressures. From top to bottom: 10 $mN \cdot m^{-1}$, 25 $mN \cdot m^{-1}$ and 35 $mN \cdot m^{-1}$.

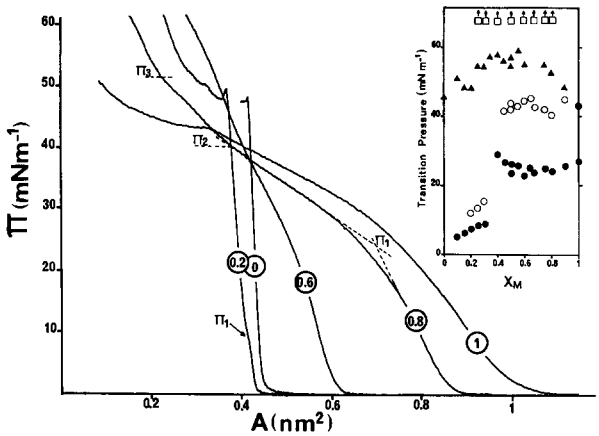

Fig. 8. Isotherm curves of mixed mycosubtilin/cholesterol monolayers. The molecular fraction x_M is indicated on the curves. Pure cholesterol (○); Pure mycosubtilin (1). Inset: transition pressure-composition phase diagram π_1 (●), π_2 (○), π_3 (▲), π_c (□).

surface pressure $\pi_1 < 10$ mN · m^{-1} (see the curve corresponding to $x_M = 0.2$ as an example) and a break point at a surface pressure called π_3 because its value (more than 46 mN · m^{-1}) is near the π_3 values defined above. Probably the break corresponding to the π_2 value is, in these cases, too discrete to be detected.

The collapse pressure π_c of the mixed monolayers is always upper than 65 mN · m^{-1} (detection limit of our apparatus).

In order to study the miscibility process the transition pressure – composition phase diagram is constructed (Fig. 8 inset). It appears that there is a discontinuity in the ($\pi_1 - x_M$) and the ($\pi_2 - x_M$) plots between $x_M = 0.3$ and 0.4. The plot of the π_3 values has an increasing part starting from the collapse pressure of cholesterol ($x_M = 0$) in the cholesterol-rich region and a decreasing one ending at the collapse pressure of mycosubtilin ($x_M = 1$) in the mycosubtilin-rich region. The ($\pi_3 - x_M$) plot is typical of the collapse pressure composition diagram of two compounds miscible in monolayer [14].

Fig. 9 shows the mean molecular area composition dependence of mixed mycosubtilin/cholesterol mono-

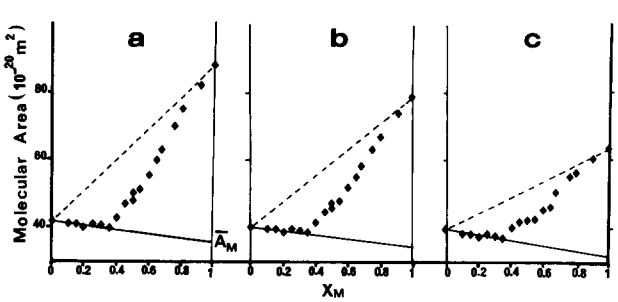

Fig. 9. Mean molecular areas of mixed mycosubtilin/cholesterol monolayers as a function of composition at various surface pressures. (a) 10 mN·m^{-1} (b) 20 mN·m^{-1} (c) 30 mN·m^{-1}. $\bar{A}_M$ is the partial molecular area of mycosubtilin when x_M is less than 0.4.

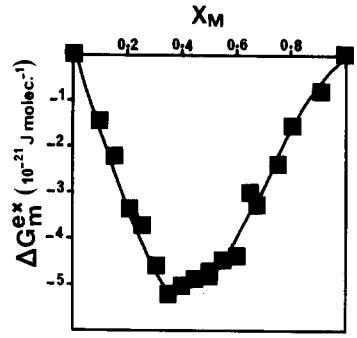

Fig. 10. Thermodynamics of the mixing process of mycosubtilin and cholesterol in monolayers. Excess free energy of mixing as a function of composition.

layers, at various pressures. These plots present negative deviations from the additivity rule. The data are distributed along two straight lines that intercept near $x_M = 0.35$. The partial molecular area of mycosubtilin when x_M is less than 0.4 is near that of pure cholesterol monolayer (the apparent reduction of mycosubtilin area is then around 60%). When x_M is comprised between 0.4 and 1, the partial molecular area of mycosubtilin is that of pure mycosubtilin monolayer, and the apparent partial molecular area of cholesterol is greatly reduced (more than 70% at 10 mN · m^{-1}).

To study the spontaneity of the miscibility process and the interactions of the molecules in the mixed films, the thermodynamic properties of the systems have been analyzed by calculating the excess free energy of mixing ΔG_m^{ex} by the relationship of Goodrich [13,15]

$$\Delta G_m^{ex} = \int_0^{\pi} A_{12}\,d\pi - x_1\int_0^{\pi} A_1\,d\pi - x_2\int_0^{\pi} A_2\,d\pi$$

where A is the mean molecular area, x the molar fraction and subscripts 1, 2 and 12 refer, respectively, to pure components 1 and 2 and to their mixtures. The upper limit of integration is 41 mN · m^{-1}.

Fig. 10 shows the variations of ΔG_m^{ex} versus the composition of the mixed mycosubtilin/cholesterol monolayers. We observe negative values on the whole range of composition with a minimum near $x_M = 0.35$.

As a comparison, we observe largely positive values of ΔG_m^{ex} for mixed mycosubtilin/DMPC monolayers specially when the mycosubtilin-DMPC complex is in the presence of an excess of DMPC (data not shown).

Discussion

The whole set of results presented here clearly indicates that mycosubtilin interacts with membrane lipids. The first evidence is the ability of mycosubtilin to induce ionic pores in planar membranes. This property is a common feature of iturin lipopeptides [9,16,17] and is related at least to the penetration of the lipopeptide molecules in the lipid bilayer [18]. The

monolayer approach gives important informations on the miscibility behaviour of mycosubtilin with DMPC and cholesterol. Mycosubtilin and DMPC are miscible in monolayer and the results show the existence of a privileged packing arrangement with a finite stoichiometry. This stoichiometry is the same (1 : 2) whatever the surface pressure. Indeed we observe a discontinuity in the mean molecular area–composition diagram always in the same range of molar fractions ($x_M = 0.35$). Furthermore we find the same stoichiometry analyzing either ($\pi_1 - x_M$) plot or ($\pi_2 - x_M$) plot. Then we can say that a 1 : 2 mycosubtilin-DMPC complex exists in monolayer. However, this complex is not miscible with DMPC since (i) the transition pressures do not vary as long as the complex is in the presence of an excess of DMPC and (ii) the positive values of the variations of the free energy of mixing indicate the formation of distinct DMPC and complex domains. On the other hand, the mycosubtilin-DMPC complex is miscible with mycosubtilin but nevertheless stays as a definite entity (i.e., there is no formation of another complex with a different stoichiometry) since the collapse pressure of the complex is the same on the whole range of composition. Therefore in the region III of the phase diagram there is coexistence between a bulk mycosubtilin and a mycosubtilin/DMPC complex monolayer.

All the results concerning mycosubtilin/cholesterol systems indicate both miscibility and non ideality. Mycosubtilin and cholesterol are miscible in monolayer in all proportions as attested by the variations of the surface transition pressures versus composition. The discontinuity observed in the ($\pi_1 - x_M$) plot near $x_M = 0.35$ as well as the change in slope in the mean molecular area–composition at the same range of molar fraction, indicate the formation of a mycosubtilin-cholesterol (1 : 2) complex. This complex is miscible both in the cholesterol and in the mycosubtilin monolayers. It seems that in the presence of an excess of cholesterol the complex adopts a peculiar arrangement where the apparent partial molecular area of mycosubtilin is very low (in the range of the area of cholesterol). This so called 'condensing' effect of cholesterol that has been observed first in mixed phospholipid/cholesterol monolayers [19] was found also in mixed iturin A/cholesterol monolayers [20]. The thermodynamic approach confirms the existence of a mycosubtilin-cholesterol complex at $x_M = 0.35$ (minimum in the $\Delta G_m^{ex} - x_M$ plot) in favor of a 1 : 2 stoichiometry.

The comparison between the mycosubtilin and the iturin A–lipid interactions are of interest to a more general view of the phenomena. Both the lipopeptides induce ionic pores in lipid membranes and although their characteristics present some differences (the mycosubtilin induced pores fluctuate more quickly) they have many common aspects, specially evolution with time, increased dwell-time in the open state when the membrane contains cholesterol and induced anionic selectivity. With regard to ionic selectivity it is interest to note that a biologically inactive compound, the methylated iturin A, induced cationic selectivity in lipid membranes [17] and the possibility of an interaction between iturins and ions is not ruled out. It had been previously suggested [20] that these ionic pores are the consequence of the presence of iturin A aggregates in the phospholipid membrane. In the case of mycosubtilin, the structures responsible of the increased permeability should not be mycosubtilin aggregates but associations of mycosubtilin-DMPC (1 : 2) complex since this complex is not miscible with DMPC. A complex with the same stoichiometry has been found between DMPC and the biologically inactive MeTyr-iturin A (and not iturin A) suggesting that the formation of iturin-phospholipid complex is not a prerequisite for the antifungal power. As a matter of fact, it is rather the presence of aggregated structures in the bilayer that seems necessary. Another important factor is the existence of priviled interactions with cholesterol and the formation of an iturin-cholesterol complex. This is true in the case of iturin A and mycosubtilin which are active compounds, but not in that of MeTyr-iturin A [20]. Mycosubtilin, the most potent compound interacts more extensively with cholesterol than iturin A. The minimum observed in the ΔG_m^{ex} values is -5×10^{-21} J · mol^{-1} for mycosubtilin and only $-2.5 \cdot 10^{-21}$ J · mol^{-1} for iturin A. On the other hand, the minimum does not occur at the same lipopeptide molar fraction and the stoichiometry of the lipopeptide-cholesterol complex is 1 : 2 for mycosubtilin and 1 : 1 for iturin A [20].

We already know that the simple inversion of two residues in the peptide sequence changes the conformation of iturins [5,6]. This study shows that this conformation change induces some differences in the iturin–lipid interactions, in their nature (the complexes formed with cholesterol have not the same stoichiometry) as well as in their intensity. On the biological point of view, the consequence is an increased antifungal activity of mycosubtilin with respect to iturin A [1]. Therefore, the biological activity of iturins is modulated by the peptide sequence either because a functional group is blocked (methylation of D-Tyr) or because a sequence change induces a conformation change.

With regard to the mechanism of action of iturins, we can propose the following schematic model: iturin molecules reach and penetrate the cytoplasmic membrane of the target cell. They disorganize the lipid bilayer by activating phospholipases in situ [21]. Inside the membrane they form oligomeric ion conducting structures (lipopeptide aggregates or lipopeptide-phospholipid complex aggregates) that are growing larger in course of time and allow the leakage of intracellular K^+. On the other hand, when active, iturins form com-

plexes with membrane sterols, therefore the biologically efficient structure may be a ternary structure: iturin/phospholipid/sterol. This scheme might applied to a great number of antifungal compounds increasing the membrane permeability as amphotericin [22–25], alamethicin [26–28]... that selfassociate on one hand, interact with phospholipids and form complexes with sterols on the other hand.

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