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Interactions of the antifungal mycosubtilin with ergosterol-containing interfacial monolayers

Mehmet Nail Nasir, Françoise Besson *

Université de Lyon, Villeurbanne, F-69622, France
Université Lyon 1, Villeurbanne, F-69622, France
INSA de Lyon, Villeurbanne, F-69622, France
CPE Lyon, Villeurbanne, F-69616, France
ICBMS CNRS UMR 5246, Villeurbanne, F-69622, France

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ABSTRACT

Mycosubtilin, an antimicrobial lipopeptide produced by *Bacillus subtilis*, is characterized by strong antifungal activities. The molecular mechanisms of its biological activities on the membranes of the sensitive yeasts or fungi have not yet been clearly elucidated. Our purpose was to mimic the mycosubtilin interactions with these membranes using various Langmuir monolayers. Since the major sterol of yeasts or fungi is ergosterol, the interactions of mycosubtilin with monolayers constituted by ergosterol, DPPC/ergosterol or DPPC/sphingomyelin/ergosterol were examined at different initial surface pressures (ΠI). Plotting the mycosubtilin-induced surface pressure increases versus ΠI allowed to determine that the exclusion pressures of mycosubtilin from these different monolayers is higher than the surface prevailing within the biological membranes. However, this behavior was lost when mycosubtilin was interacting with ergosteryl acetate-containing monolayers. This suggests the involvement of the sterol alcohol group in the mycosubtilin interactions within membranes. Furthermore, the behavior of mycosubtilin with stigmasterol, similar to that observed with ergosterol, differs from that previously observed with cholesterol, suggesting a role of the alkyl side chain of the sterols. The adsorption of mycosubtilin to ergosterol monolayers induced changes in the lipopeptide orientation at the air–water interface as revealed by polarization modulation infrared reflection absorption spectroscopy (PM-IRRAS). Moreover, imaging the air–water interface by Brewster angle microscopy (BAM) indicates that mycosubtilin induced changes in the organization and morphology of monolayers containing pure ergosterol with the appearance of small condensed dots, suggesting again that the target of mycosubtilin might be the ergosterol present in the membranes of the sensitive yeasts or fungi.

1. Introduction

Mycosubtilin has been identified as an iturinic antifungal compound in the culture medium of several strains of *Bacillus subtilis* [1,2]. It is constituted by a heptapeptide, cyclized in a ring with a β-amino fatty acid (Fig. 1) [3]. The β-amino fatty acid chain can be *iso* C_{16}, *n* C_{16}, *iso* C_{17} or *anteiso* C_{17} [4]. Mycosubtilin exhibits biocide activities [5] for some pathogenic strains which are resistant to classical antifungal agents [6]. Among the mycosubtilin-sensitive strains are *Botrytis cinerea*, *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, *Cryptococcus neoformans*, *Fusarium oxysporum*, *Pichia pastoris*, *Saccharomyces cerevisiae* and *Yarrowia lipolytica*. Furthermore, the cell target of the iturinic lipopeptides has been studied using *S. cerevisiae* as model yeast and identified as the cytoplasmic membrane [5,7,8].

In a recent work, the molecular mechanisms of the hemolytic activity of mycosubtilin were analyzed by examining its interactions with monolayers constituted by pure lipids (DPPC, cholesterol or sphingomyelin) or by lipid mixtures (DPPC/cholesterol or DPPC/cholesterol/sphingomyelin) [9]. It has been shown that mycosubtilin induced the most striking changes in the organization and morphology of the monolayers only when they contained the DPPC, cholesterol and sphingomyelin, monolayers which could be considered to mimic the lipid rafts [10]. Furthermore, cholesterol has been identified as the potential cellular target of mycosubtilin [9] but this cannot explain the antifungal activities of mycosubtilin since the cytoplasmic membranes of yeasts or fungi do not contain significant amounts of cholesterol. Indeed the major sterol of their plasma membrane is ergosterol [11,12]. Ergosterol differs from cholesterol by the presence of two additional double bonds,

Abbreviations: BAM, Brewster angle microscopy; Chol, cholesterol; DPPC, dipalmitoylphosphatidylcholine; Erg, ergosterol; GL, gray level; ΠEx, exclusion pressures; Π_i, initial surface pressure; OS, obturation speed; PM-IRRAS, polarization modulation infrared reflection absorption spectroscopy

* Corresponding author at: ODMB, ICBMS CNRS UMR 5246, Université Lyon 1, Bâtiment Raulin, 43 boulevard du 11 novembre 1918, F-69622 Villeurbanne Cedex, France. Tel.: +33 4 26 23 44 13; fax: +33 4 72 43 15 43.
E-mail address: f-besson@univ-lyon1.fr (F. Besson).

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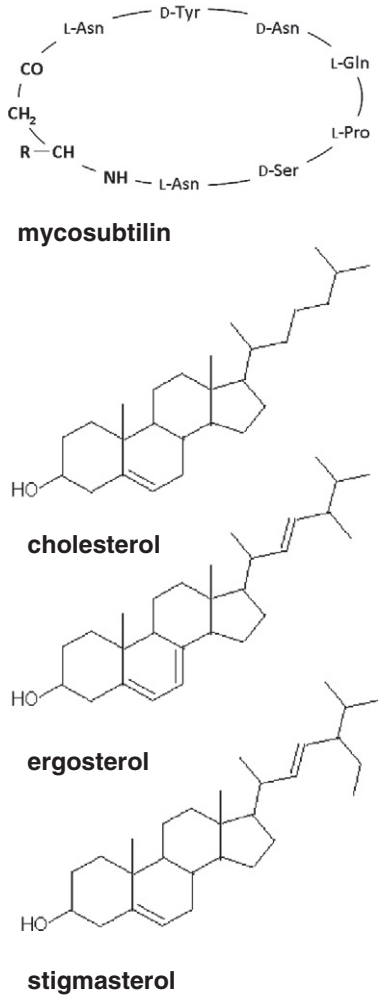

Fig. 1. Schematic structures of mycosubtilin and different sterols.

one in the steroid nucleus and the other one in the alkyl side chain, as well as a by the presence of an extra methyl group in this side chain (Fig. 1).

Furthermore the antifungal activities of mycosubtilin have been shown to be inhibited by adding ergosterol to the culture medium of the sensitive cells [6,13], suggesting an interaction between the lipopeptide and ergosterol. The present work is the first attempt to explain the biocide activities of mycosubtilin by looking for the role played by ergosterol in its antifungal power. We also addressed the following question: does mycosubtilin bind preferentially to the lipid rafts of the sensitive cells which are supposed to contain phosphatidylcholine, sphingomyelin and ergosterol? The interactions between mycosubtilin and the cytoplasmic membranes of yeasts or fungi were modeled by using biomimetic Langmuir monolayers at the air–water interface, as it had been done for amphotericin B, another antifungal molecule [14]. It was shown that the nature of the sterols plays a role in the interaction of amphotericin B, we have chosen to analyze the adsorption of mycosubtilin to monolayers constituted by stigmasterol, another sterol.

In the present work, the orientation of the lipopeptide at the air–water interface was analyzed by polarization modulation infrared reflection absorption spectroscopy (PM-IRRAS) which is sensitive to protein or peptide conformations and to the mean orientation of the transition dipole. The morphology of the mycosubtilin/lipid complexes was analyzed by Brewster angle microscopy (BAM), an interfacial technique detecting the presence of condensed domains in monolayers.

2. Materials and methods

2.1. Chemicals

Bovine brain sphingomyelin (SM), dimethylsulfoxide (DMSO), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), ergosterol (Ergo) and stigmasterol were purchased from Sigma Chemical Co. (St. Louis, MO). Ergosteryl acetate (Ergo-Ac) was purchased from Sobioda (Montbonnot St Martin, France). They were used without further purification. All organic solvents were of analytical grade. The ultrapure water, purified with a Millipore filtering system (Bedford, MA), had a resistivity of 18.2 MΩ cm. Stock solutions of the lipids were obtained by dissolving them at 0.545 mM in hexane–ethanol (9/1, v/v). DPPC/Ergo and DPPC/Ergo/SM solutions were obtained by mixing the stock solutions at the desired mole ratio. The purity of mycosubtilin, prepared from cultures of *B. subtilis* strain, was checked by thin-layer chromatography [15], and it was about 98%. Stock solution of mycosubtilin was obtained by dissolving it into DMSO at 0.545 mM [16].

2.2. Adsorption experiments at constant surface area

The film balance was built by R&K (Riegler & Kirstein GmbH, Wiesbaden, Germany) and equipped with a Wilhelmy-type surface-pressure-measuring system. All monolayer experiments were performed at 21 °C. Adsorption experiments were performed on a small Teflon dish (diameter, 4 cm; volume, 10 mL) with an ultrapure water subphase (pH 5.6). The subphase was stirred with a magnetic stirrer spinning at 100 rev. /min. Lipids were spread at the air–water interface to reach the desired final surface pressure. After solvent evaporation and film stabilization [17], mycosubtilin was injected at a final concentration of 0.545 μM into the subphase. The lipopeptide adsorption to the different lipid monolayers was measured as an increase in the surface pressure as described previously [9,18]. To determine whether DMSO was not interfering with the surface chemistry properties, the same volume of pure DMSO was injected under the lipid monolayer as control, and no change in surface pressure was detected as previously shown [9,16].

2.3. PM-IRRAS measurements

PM-IRRAS is an IR spectroscopic method adapted to the air–water interface. It combines Fourier transform mid-IR reflection spectroscopy with rapid polarization of the incident beam between parallel (p) and perpendicular (s) polarization [19–21]. The spectra were recorded on a Nicolet 850 spectrometer equipped with an HgCdTe detector which was cooled down at −196 °C with liquid nitrogen (Thermo Electron, Nicolet Instrument, Madison, WI). In short, the infrared beam was reflected toward the optical bench by a mirror. The reflected beam was then polarized by a ZnSe polarizer and modulated between a parallel (p) and a perpendicular (s) polarization by a photoelastic modulator. The polarized and modulated beam was then directed toward the air–water interface onto a small Langmuir trough (Nima Technology, UK) as described previously [22]. The optimal angle of incidence was 75° to the interface normal. Afterwards, the beam was reflected on the HgCdTe detector. The detected signal allowed obtaining the differential reflectivity spectrum after processing as follows:

$$\Delta R/R = \left[\left(R_p - R_s\right) J_2(\Phi_0)/\left(R_p + R_s\right) + \left(R_p + R_s\right) J_0(\Phi_0)\right],$$

where J_2 and J_0 are the zero- and second-order Bessel functions, while Φ_0 is the phase of the Bessel functions related to variations of the modulation of polarization according to the wavelength and R_p and R_s are the parallel and perpendicular reflectivity,