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Native and dry-heated lysozyme interactions with membrane lipid monolayers: Lipid packing modifications of a phospholipid mixture, model of the *Escherichia coli* cytoplasmic membrane

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ABSTRACT

Antimicrobial resistance is currently an important public health issue. The need for innovative antimicrobials is therefore growing. The ideal antimicrobial compound should limit antimicrobial resistance. Antimicrobial peptides or proteins such as hen egg white lysozymes are promising molecules that act on bacterial membranes. Hen egg white lysozymes have recently been identified as active on Gram-negative bacteria due to disruption of the outer and cytoplasmic membrane integrity. Furthermore, dry-heating (7 days and 80 °C) improves the membrane activity of lysozymes, resulting in higher antimicrobial activity. These *in vivo* findings suggest interactions between lysozymes and membrane lipids. This is consistent with the findings of several other authors who have shown lysozyme interaction with bacterial phospholipids such as phosphatidylglycerol and cardiolipin. However, until now, the interaction between lysozyme and bacterial cytoplasmic phospholipids has been in need of clarification. This study proposes the use of monolayer models with a realistic bacterial phospholipid composition in physiological conditions. The lysozyme/phospholipid interactions have been studied by surface pressure measurements, ellipsometry and atomic force microscopy. Native lysozyme has proved able to absorb and insert into a bacterial phospholipid monolayer, resulting in lipid packing reorganization, which in turn has lead to lateral cohesion modifications between phospholipids. Dry-heating of lysozymes has increased insertion capacity and ability to induce lipid packing modifications. These *in vitro* findings are then consistent with the increased membrane disruption potential of dry heated lysozyme *in vivo* compared to native lysozyme. Moreover, an eggPC monolayer study suggested that lysozyme/phospholipid interactions are specific to bacterial cytoplasmic membranes.

1. Introduction

The discovery of new antimicrobial molecules is greatly necessary as a means to counterbalance the prominent public health problem of antimicrobial resistance [1]. In order to limit the development of bacterial resistance, peptides or proteins which target the bacterial cell membranes should be considered as relevant antimicrobial molecules. These antimicrobial peptides or proteins generally permeate the bacterial outer and/or cytoplasmic membranes and their movement leads to bacterial cell death [2].

The hen egg white lysozyme is one of the antimicrobial proteins that has been widely used in pharmaceutical applications. This protein is historically known for its enzymatic hydrolysis of peptidoglycan, causing its antimicrobial activity on Gram-positive bacteria [3]. However, this protein is more than just an enzyme; it is also able to disrupt the bacterial membranes, to inhibit the synthesis of DNA or RNA and to induce autolysin production [4–7]. Hence, lysozyme is active against both Gram-negative and Gram-positive bacteria [4,7]. In particular, it has been recently established that lysozyme permeates both the outer and inner membranes of *Escherichia coli*, respectively with and without perforations [6,7]. Moreover, lysozyme depolarizes the cytoplasmic membrane and causes cytosol leakage [7]. However, the antimicrobial effect of lysozymes on Gram-negative species remains limited [4,7].

Abbreviations: AMP, antimicrobial peptide or protein; CA, cardiolipin; CMEC, *Escherichia coli* cytoplasmic phospholipid mixture; DH-L, dry-heated lysozyme; DOPE, 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine; DOPG, 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); DPPE, 1,2-dihexadecanoyl-*sn*-glycero-3-phospho-ethanolamine; DPPG, 1,2-dihexadecanoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); EggPC, hen egg L-α-phosphatidylcholine; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; LC, liquid condensed; LE, liquid expanded; N-L, native lysozyme

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Dry-heating, a common practice to decontaminate pharmaceutical products, has been shown to improve lysozyme antimicrobial activity on *E. coli* [7–9]. A higher membrane disruption potential is at the basis of this improvement. Increased protein–membrane interactions are due to the more favorable physicochemical characteristics of dry-heated lysozymes [7]. Dry-heating lysozymes result in succinimide derivatives, making dry-heated lysozymes more basic and more flexible; these chemical modifications also induce higher surface hydrophobicity [10–13].

These *in vivo* findings thus indicate that lysozymes interact with bacterial membrane lipids. To investigate lysozyme–lipid interactions and the impact thereof on the bacterial membrane, *in vitro* models such as lipid monolayers can be used. Interfacial monolayers are considered as good models to evaluate protein–lipid interactions, since the initial lateral lipid pressure can be controlled and multiple lipid compositions can be used [14,15]. In this context, our group had previously investigated the lysozyme–lipopolysaccharide interactions in order to have a better understanding of the lysozyme outer membrane disruption. We established that it was possible to insert lysozymes into LPS monolayers, a model for the *E. coli* outer membrane, and reorganize this lipid film laterally and vertically [16]. In this study, the aim is to investigate lysozyme interactions with bacterial cytoplasmic membrane phospholipids. Previous studies established that the lysozyme–phospholipid interactions are highly dependent on the pH, ionic strength and lipid nature [17–20]. To obtain a maximal significance from the results, environmental conditions and phospholipid composition should be as close as possible to the natural situation. In particular, the use of a complex lipid mixture is key, due to the complexity of electrostatic and hydrophobic interactions between the different phospholipids, and due to the impact of the lipid geometry and structure on the later lipid packing [21]. Thus, lysozyme interactions with bacterial cytoplasmic lipids were studied here for the first time in physiological conditions (pH 7 and ionic strength of 155 mM) using a phospholipid monolayer constituted of a lipid mixture close to the natural *E. coli* K12 composition as described by Lugtenberg et al. (1976) [22].

Since electrostatic and hydrophobic interactions are considered as the major interactive forces between lysozyme and the phospholipids [18,19], the comparison between native and dry-heated lysozymes could possibly reveal interesting differences. Indeed, dry-heating of lysozymes induces physicochemical modifications as mentioned above. These modifications should enhance lysozyme–phospholipid interactions such as protein adsorption and insertion, explaining the increased membrane depolarization and ion channel formation observed *in vivo* after dry-heating [7].

In this study, interactions between lysozymes and the phospholipid monolayer membrane model were investigated using biophysical tools such as ellipsometry, surface pressure measurements and atomic force microscopy (AFM).

2. Material and methods

2.1. Proteins and lipids

Native lysozyme (N-L) powder (pH 3.2) was obtained from Liot (Annezin, 62-France). It was heated for 7 days at 80 °C in hermetically closed glass tubes to obtain dry-heated lysozyme (DH-L). Lysozyme (N-L or DH-L) was solubilized (around 0.5 g/L) in 5 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (Sigma Aldrich, Saint-Quentin, France) pH 7.0 with 150 mM NaCl (Fluka, Saint-Quentin, France). The concentration of the lysozyme stock solution was precisely determined by absorbance at 280 nm (extinction coefficient = 2.6 $g^{-1}\cdot L$) [23]. The protein solution was then diluted in the HEPES buffer to obtain the desired lysozyme concentration.

A mixture of different lipids (Avanti Polar Lipids, Alabaster, USA) was prepared in order to obtain a composition close to the natural one present in the cytoplasmic membrane of *E. coli* as detected by Lugtenberg et al. [22]; it contained 2.6% 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1′-rac-glycerol) (DOPG), 3.9% 1,2-dihexadecanoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DPPG), 11.8% cardiolipin (CA), 32.3% 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (DOPE) and 49.4% 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DPPE). This lipid mixture (CMEC) was prepared in 2:1 chloroform/methanol mixture at 0.25 mM. Hen egg L-α-phosphatidylcholine (eggPC) (Avanti Polar Lipids, Alabaster, USA) was also prepared in 2:1 chloroform/methanol mixture at 1 mM.

2.2. Lipid/protein monolayers

The experiments were performed in a homemade TEFLON® trough of 8 mL at 21 °C. Before each use, the trough was thoroughly and successively cleaned with warm tap water, ethanol and ultra-pure water, then boiled for 15 min in ultra-pure water. After cooling the TEFLON® trough, it was filled with an 8 mL HEPES buffer. The CMEC was spread with a high precision Hamilton microsyringe at the clean air/liquid interface to obtain an initial surface pressure of 20 ± 1 or 30 ± 1 mN/m. The eggPC was spread as described for the CMEC to obtain an initial surface pressure of 30 mN/m. After 15 min, *i.e.* a duration necessary to allow solvent evaporation and lipid organization, 50 μL N-L or DH-L solution was injected into the subphase with a Hamilton syringe in order to obtain a final protein subphase concentration between 0.02 and 3 μM.

2.3. Surface pressure measurements

The surface pressure was measured following a Wilhelmy method using a 10 mm × 22 mm filter paper as plate (Whatman, Velizy-Villacoublay, France) connected to a microelectronic feedback system (Nima PS4, Manchester, England). The surface pressure (π) was recorded every 4 s with a precision of ±0.2 mN/m. The measured surface pressure was the result of the surface tension of water minus the surface tension of the lipid film.

2.4. Ellipsometry

Measurements of the ellipsometric angle value were carried out with an in-house automated ellipsometer in a "null ellipsometer" configuration [24,25]. A polarized He–Ne laser beam (λ = 632.8 nm, Melles Griot, Glan-Thompson polarizer) was reflected on the liquid surface. The incidence angle was 52.12°, *i.e.* Brewster angle for the air/water interface minus 1°. After reflection on the liquid surface, the laser light passed through a λ/4 retardation plate, a Glan-Thompson analyzer, and a photomultiplier. The analyzer angle, multiplied by two, yielded the value of the ellipsometric angle (Δ), *i.e.* the phase difference between parallel and perpendicular polarization of the reflected light. The laser beam probed the 1 mm^2 surface with a depth in the order of 1 μm. Initial values of the ellipsometric angle (Δ_0) and surface pressure (π_0) of buffer solutions were recorded for at least half an hour to assure that the interface was clean. Only in the case of a stable and minimal signal were experiments performed. Values of Δ were recorded every 4 s with a precision of ±0.5°.

2.5. AFM sample preparation and AFM imaging

Experiments were performed with a computer-controlled and user-programmable Langmuir TEFLON®-coated trough (type 601BAM) equipped with two movable barriers and of total surface 90 cm^2 (Nima Technology Ltd., England). Before starting the experiments, the trough was cleaned successively with ultrapure water (Nanopure-UV), ethanol, and finally ultrapure water. The trough was filled with a 5 mM HEPES buffer pH 7 with 150 mM NaCl. CMEC was spread over the clean air/liquid interface at a surface pressure of 20 ± 1 mN/m. The solvent was then left to evaporate for 15 min. Then, a Langmuir–

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