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Effect of phenol-induced changes in lipid composition on conformation of OmpF-like porin of *Yersinia pseudotuberculosis*

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ABSTRACT

The present work aimed to compare the effects of different lysophosphatidylethanolamine (LPE) content in lipids derived from *Yersinia pseudotuberculosis* cells exposed and not exposed to phenol on the conformation of OmpF-like porin of these bacteria. Differential scanning calorimetry and intrinsic protein fluorescence showed that the 2.5-fold increase of LPE content and the corresponding increase in the phase transition temperature of bacterial lipids were accompanied by enhanced protein thermostability. Integral conformational rearrangement of protein was supported by drastic changes in the microenvironment of the tryptophan residues, likely resulting in a convergence of monomers in trimeric porin and exposure of outer tryptophan residues to the water environment. These conformational changes may impede the porin channel permeability under stress conditions in bacteria.

1. Introduction

The lipid bilayer of biological membranes is characterized by complex structural and dynamical properties, which are important for the structural organization and function of membrane proteins and for many cell functions. The OmpF-like porin of the Gram-negative bacteria *Yersinia pseudotuberculosis* (YOmpF) is a cylinder-shaped homotrimeric protein that forms a non-specific transmembrane channel, allowing passive diffusion of hydrophilic low-molecular-mass substances across the bacterial outer membrane [1]. Thus, the protein–lipid interactions are crucial for cellular functions of this protein.

Membrane lipids form a dynamic liquid crystalline matrix, the fluidity of which is maintained under altering environmental conditions of bacteria and other poikilothermic organisms [2]. It is assumed that compensatory changes in physicochemical properties of membrane lipids are necessary to optimize functional properties of membrane proteins under new environmental conditions [3,4]. The absence of effective adjustments to environmental stresses results in deterioration in cellular functions or in cell death of poikilothermic organisms [5].

In spite of the high ability of *Y. pseudotuberculosis* to adapt to different environmental conditions by changing its lipid composition, the effect of different lipid surroundings on the conformation of the major membrane protein – YOmpF porin – is still unclear. However, our study of the properties of the hybrid system, comprising glycolipid monogalactosyldiacylglycerol from different marine macrophytes and YOmpF led to the conclusion that physicochemical properties of lipid surroundings are very important for conformation and immunogenicity of this protein [6].

Various stress factors such as heat shock [7], low pH of medium [8] and treatment by phenol biocide [9] were shown to drastically increase the content of lysophosphatidylethanolamine (LPE), the level of which usually does not exceed 2–5% in membranes of bacteria. Accumulation of LPE in bacterial membranes is supposed to be one of a number of adaptive responses to stress.

The present work is aimed to study the effect of unusual accumulation of LPE in total lipids of *Y. pseudotuberculosis* cells exposed to phenol on the conformation of OmpF-like porin of these bacteria. Since this main adaptive response, namely conversion of outer membrane PE to LPE catalyzed by phospholipase A,

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occurs in outer membrane, where porin localizes also, isolated total lipids were used to model lipid microenvironment of purified porin in vitro.

2. Materials and methods

2.1. Bacterial cells

The strain KS 3058 of *Y. pseudotuberculosis O*:Ib serovar was cultivated in nutrition broth (NB, Obolensk, Russia) at 8 °C. Bacterial cells were grown in 1-L flasks for 6 days. After achievement of early stationary phase [10], bacteria were either treated by 1% phenol for 20 min or were left intact. Bacterial cells were separated from culture medium by centrifugation at 5000 rpm for 20 min. The separated cells were washed two times in physiological solution (0.85% NaCl, pH 5.5).

2.2. Isolation and thin-layer chromatography of lipids

Total lipids were extracted twice by chloroform–methanol (2:1, by volume) for 2 h at 8 °C from bacterial cells. The suspension of cells was centrifuged at 3000 rpm for 15 min. Supernatant (the first lipid extract) was discarded, and the pellet was re-extracted with the chloroform–methanol mixture. Freshly prepared lipid extracts were used for the following procedures.

Phospholipid (PL) composition was defined by thin-layer chromatography (TLC) using chloroform–methanol–benzene-28% NH_4-OH (65:30:10:6, by volume) in the first direction and chloroform–methanol–acetic acid–acetone–benzene–water (70:30:4:5:10:1, by volume) in the second direction. PLs were identified by comparison with authentic samples and by specific reagents. Non-specific detection of PL was carried out with 10% H_2SO_4 dissolved in methanol, followed by heating at 180 °C for 10 min.

2.3. Gas–liquid chromatography

Fatty acid methyl esters were analyzed by gas–liquid chromatography (GLC). Methylation was performed with 5% HCl in methanol for 1 h at 90 °C. Methyl esters of fatty acids were purified by TLC on silica gel with benzyl mobile phase. GLC analysis was performed by Agilent 6890 GC gas chromatograph at the isothermal regime with INNOWax capillary column (25 m × 0.25 mm × 0.25 μm).

The evaporator temperature was 240 °C; the column and detector temperatures were maintained at 200 °C and 250 °C, respectively. Helium was used as the carrier gas with a linear velocity of 35 cm s^{-1}. Fatty acids were identified according to their equivalent chain-length (ECL).

2.4. Preparation of lipid–porin complexes for DSC and spectroscopic studies

Trimeric YOmpF porin was isolated from *Y. pseudotuberculosis* (stain 598, serovar 1B) according to [11]. Purity of porin sample was confirmed by SDS–PAGE and N-terminal analysis [12]. Pore-forming activity of the protein was established by BLM technique [13,14]. Total lipids from *Y. pseudotuberculosis* dissolved in chloroform were introduced into standard aluminum pans. Vacuum-dried lipid samples of 1 mg were vortexed in 100 μl of 0.03 M Tris–Cl buffer, pH 7.8, and preheated to approximately 50 °C. Then, 0.5 mg of porin solubilized in 0.03 M Tris–Cl buffer, pH 7.8, with 0.125% *n*-octyl-β-D-glucopyranoside was added and mixed with the lipid dispersion by vortex. As found earlier, *n*-octyl-β-D-glucoside maintains the YOmpF conformation as close to its spatial structure in the membrane and does not disintegrate bilayer lipid membrane [11]. The resulting suspension was left for 2 h and was then used to study protein conformation by calorimetric and spectroscopic methods.

For additional method, see Supplementary Materials and Methods.

2.5. Calorimetry

Total lipids from *Y. pseudotuberculosis* were solubilized in chloroform and introduced into standard aluminum pans. Vacuum-dried samples of approximately 5 mg each were mixed with a twofold quantity of water–ethylene glycol (2:1, by volume), and sealed into pans, which then were placed in a DSC-2M differential scanning calorimeter (Puschino, Russia). Samples were either heated or cooled at 16 °C min^{-1} between −100 and 60 °C at a sensitivity of 40 mW. Position of the maximum of heat capacity *vs.* temperature plot was taken for the phase transition temperature, T_{max}. The temperature range was calibrated by using naphthalene, mercury, and indium.

Calorimetric experiments with porin were performed on a Scal-1 differential scanning microcalorimeter (Scal Co., Ltd., Pushchino, Russia), as described previously [6]. Before measurement, the sample and reference solutions were thoroughly degassed and carefully loaded into the cells to avoid bubble formation. Exhaustive cleaning of the cells was undertaken before each experiment. An overpressure of 2 atm was always kept over the liquids in the cells throughout the scans to prevent any degassing during heating. A background scan collected with buffer in both cells was subtracted from each scan. The reversibility of the thermal transitions was checked by examining the reproducibility of the calorimetric trace in a second heating of the sample immediately after its cooling from the first scan. The calorimeter was interfaced with a personal computer and the data were acquired and processed by SCAL software.

Analysis of the data was accomplished on the basis of a simple two-state irreversible model, $N_3 \xrightarrow{k} D_3$, where N_3 and D_3 are native and denatured porin trimers, respectively, and k is the effective rate constant for denaturation changes with temperature, as given by the Arrhenius equation: $k = \exp\{E_A(1/T^* - 1/T)/R\}$, where E_A is the energy of activation, R is the gas constant, and T^* is the temperature at which the rate constant equals 1 min^{-1}. In this case the excess heat capacity C_p^{ex} is given by the following equation [15]:

$$C_p^{ex} = \frac{1}{v}\Delta H \exp\left\{\frac{E_A}{R}\left(\frac{1}{T^*} - \frac{1}{T}\right)\right\} \times \exp\left\{-\frac{1}{v}\int_{T_0}^{T} \exp\left[\frac{E_A}{R}\left(\frac{1}{T^*} - \frac{1}{T}\right)\right] dT\right\} \quad (1)$$

where $v = dT/dt$ (K min^{-1}) is a scan rate value and ΔH is the enthalpy difference between the denatured and native states.

2.6. Fluorescence spectroscopy

Steady-state fluorescence measurements were carried out on a PC1 spectrofluorimeter at 25 °C (ISS, USA). Excitation was performed at 296 nm (with excitation and emission slit widths of 3 nm). Fluorescence was measured in the range of 300–400 nm. Porin fluorescence measurements were carried out in protein solutions with an optical density of less than 0.2 at 280 nm in order to avoid the inner filter effect. Emission spectra were corrected for baseline and instrumental spectral sensitivity.

3. Results

3.1. Characteristic of lipids from Y. pseudotuberculosis

As shown in Table 1, lipids from bacterial cells untreated by phenol contained a comparably low amount of LPE. Phenol