Biochimica et Biophysica Acta 1828 (2013) 2173–2181

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Cord factor (trehalose 6,6′-dimycolate) forms fully stable and non-permeable lipid bilayers required for a functional outer membrane

Parthasarathi Rath [a,b], Olivier Saurel [a,b], Georges Czaplicki [a,b], Maryelle Tropis [a,b], Mamadou Daffé [a,b], Alexandre Ghazi [c], Pascal Demange [a,b,*], Alain Milon [a,b,*]

[a] Institute of Pharmacology and Structural Biology, Université de Toulouse, UPS, 205 route de Narbonne, 31077 Toulouse, France
[b] IPBS, UMR 5089, CNRS, 205 route de Narbonne, BP 64182, 31077 Toulouse, France
[c] Université Paris Sud-11, CNRS UMR 8619, Institut de Biochimie et Biophysique Moléculaire et Cellulaire, 91405 Orsay, France

ARTICLE INFO

Article history:
Received 28 February 2013
Received in revised form 22 April 2013
Accepted 23 April 2013
Available online 1 May 2013

Keywords:
Mycolic acid
Cardiolipin
Ion channel
Phase transition
Molecular dynamics simulation
^{2}H NMR

ABSTRACT

Cord factor (trehalose 6,6′-dimycolate, TDM) is the major lipid in the outer membrane of *Corynebacteria* and *Mycobacteria*. Although its role is well recognized in the immune response phenomena, its membrane biophysical properties remained largely unexplored and TDM has often been described as a detergent. We purified the main components of the outer membrane from *Corynebacterium glutamicum* and analyzed their membrane forming properties. In mixture with endogenous cardiolipin, but not alone, the spontaneous hydration of TDM produces liposomes. As a pure component, TDM formed vesicles only by the detergent dialysis method. Perdeuterated cardiolipin–TDM mixtures were shown by deuterium nuclear magnetic resonance (NMR) to exhibit a gel to liquid crystalline phase transition over a 273–295 K temperature range, for cells grown at 303 K, and thus to be in a liquid crystalline state at physiological temperature. Molecular dynamics simulations of hydrated TDM bilayers provided the trehalose average orientation and conformation, the chain order parameters, the area per lipid and the bilayer thickness which was confirmed by electron microscopy. Finally the Porin A–Porin H ion channel from the *Corynebacterial* outer membrane was reconstituted in TDM liposomes. With properly mycoloylated proteins, it manifested the typical voltage dependent ion channel properties of an outer membrane porin.

1. Introduction

The cell envelope organization and properties of the group of bacteria in the suborder *Corynebacteriales* (which includes *Corynebacteria* and *Mycobacteria*) may explain why specific long chain fatty acids are indispensable for the survival of these organisms. These bacteria present a unique cell envelope that contains an outer membrane, also called the mycomembrane, which provides a permeability barrier resembling that of Gram-negative bacteria. The mycomembrane consists of a bilayer composed of an inner leaflet of mycolic acids covalently linked to the cell wall arabinogalactan, which in turn is attached to peptidoglycan, and an outer leaflet of various non-covalently linked lipids, including mycolic acid-containing glycoconjugates such as trehalose mono- and di-mycolate [1,2].

Trehalose 6,6′-dimycolate (TDM) is an abundant surface glycolipid in the mycomembranes which provide a potent biological barrier. TDM is also known as "cord factor" as it facilitates cord formation, as well as increasing impermeability and resistance to: (i) many antibiotics; (ii) susceptibility to acid- and alkali-induced cytotoxicity; (iii) osmotic lysis; and (iv) lethal oxidation, thus improving survival inside macrophages [3–6]. TDM also plays a major role in the immunological response and in *Mycobacteria*–host interactions [7,8]. TDM comprises a molecule of trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) esterified by two α-alkyl, β-hydroxy fatty acids (mycolic acids) of varying chain lengths: 70–90 carbon atoms in *Mycobacteria*; 46–90 carbon atoms in *Nocardia*; and 22–38 carbon atoms in *Corynebacteria* [9,10]. Other lipids have been associated with the mycomembrane, such as phospholipids and, in mycobacteria, species-specific lipids [11–14].

Recently the exact lipid composition of the outer membrane of *Corynebacterium glutamicum* has been reassessed independently by two groups. First, Bansal-Mutalik and Nikaido [15], using reverse surfactant micelles extraction, proposed that the outer membrane contains cardiolipin tightly associated with the peptidoglycan–arabinogalactan complex in the inner leaflet, and predominantly TDM in the outer leaflet. Then, Marchand et al. isolated the mycomembrane by flotation

Abbreviations: OG, *n*-octyl-β-D-glucopyranoside; PG, Phosphatidylglycerol; PI, Phosphatidylinositol; PIMs, Phosphatidylinositol-mannosides; CL, cardiolipin; TDM, trehalose 6,6′-dimycolate; TMM, trehalose-monomycolate; PorA–PorH, major outer membrane protein complex from *Corynebacterium glutamicum*; TOF, time-of-flight; EM, electron microscopy; DLS, dynamic light scattering; MD, molecular dynamics

* Corresponding authors at: IPBS, UMR 5089, CNRS, 205 route de Narbonne, BP 64182, 31077 Toulouse, France. Tel.: +33 561175423.
E-mail addresses: rathparthasarathi@gmail.com (P. Rath), olivier.saurel@ipbs.fr (O. Saurel), cgeorge@ipbs.fr (G. Czaplicki), maryelle.tropis@ipbs.fr (M. Tropis), mamadou.daffe@ipbs.fr (M. Daffé), alexandre.ghazi@biomemb.u-psud.fr (A. Ghazi), alain.milon@ipbs.fr (A. Milon), pascal.demange@ipbs.fr (P. Demange).

0005-2736/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamem.2013.04.021

density gradient, and analyzed its lipid and protein composition extensively [16]. Based on this separation protocol, the predominant lipid was identified as TDM. Both studies indicated the major role played by TDM in the physical organization of the outer membrane, either alone or in mixture with cardiolipin.

Although a significant quantity of biophysical data has been accumulated over the last 5 decades regarding the spontaneous self-assembly of many membrane lipids and their corresponding membrane physical properties (see for instance [17] and references therein), including cardiolipin [18], surprisingly, very little is known about the self-organization of TDM and/or TDM/cardiolipin lipid mixtures. The term "TDM micelle", which seems contradictory with being the major component of an impermeable outer membrane, is often found in the literature, however this phrase usually refers to TDM that has been solubilized in Freund's incomplete adjuvant, or in other detergent mixtures [5,8]. The interaction of TDM with preformed lipid bilayers has been studied, and it was shown to inhibit Ca^{2+}-induced fusion between phospholipid vesicles [19], and to affect bilayer water permeability and the electrical capacitance of phosphatidylcholine bilayers [20]. Finally, the influence of mycolic acid chain length on the phase properties of *Corynebacteriales* cell walls has been assessed, and differential scanning calorimetry was used to demonstrate that a phase transition occurs at 60–70 °C for the longer chains of *Mycobacteria*, while *Corynebacteria* displayed a transition at ~30 °C, indicating a clear correlation between mycolic acid structure and the fluidity (and thus the permeability) of mycomembranes [21].

The data described above prompted us to extract and purify TDM from *C. glutamicum* cell walls and to analyze its membrane forming properties. After characterization of the distribution of mycolic chain lengths by MALDI-TOF mass spectrometry, novel protocols to form vesicles with pure TDM or with TDM/cardiolipin mixtures were established. Pure TDM did not spontaneously form vesicles following direct hydration of the dry lipid film with excess water, but mixtures of TDM and cardiolipin (TDM:CL ratios from 1:4 to 4:1) did form vesicles in the same conditions, and these vesicles were characterized using a range of biophysical techniques. In order to form vesicles with TDM in the absence of cardiolipin, it was necessary to use the detergent dialysis method, and TDM vesicles produced in this manner were characterized by TEM, DLS and NMR spectroscopy. *C. glutamicum* was also cultured in D_2O, enabling the purification of perdeuterated TDM and cardiolipin. Deuterium NMR was then used to assess the lipid order parameters, and a gel to liquid crystalline phase transition in cardiolipin–TDM mixture was observed at a temperature just a couple of degrees below the cell growth temperature. Furthermore, the first MD simulations of hydrated TDM bilayer were carried out, and provided a detailed atomic dynamical description of the bilayer. Finally pure TDM liposomes were demonstrated to be suitable for the functional reconstitution of an ion channel from the outer membrane of *C. glutamicum*: the PorA–PorH complex. These observations clearly establish for the first time that TDM, both alone and in mixture with cardiolipin, can self-organize into a stable, non-permeable lipid bilayer required for a functional outer membrane.

2. Material and methods

2.1. Extraction and purification of TDM from C. glutamicum

C. glutamicum strain ATCC 13032 was used for extraction and purification of trehalose dimycolate. The total cell lipid extracts were obtained as described previously [22,23]. Briefly, from a primary culture, *C. glutamicum* cells were grown to late exponential phase (16 h) in brain heart infusion (BHI, Difco) media at 30 °C with continuous shaking at 200 rpm. Perdeuterated cultures were performed using BHI media in D_2O (99.8%, Eurisotop). Cells were harvested by centrifugation at 4000 *g* for 20 min. The cell wall was extracted three times successively in $CHCl_3$:CH_3OH (1:2, 1:1 and 2:1 [vol/vol]) proportions (12 ml per gram of cell mass) for 12 h at room temperature with continuous stirring. The organic solvent extracts were separated after each extraction step by centrifugation at 4000 *g* at room temperature. The insoluble material was eliminated by filtration through a Whatman paper. All organic solvents were removed by vacuum rotary evaporator. The organic extract was dissolved in $CHCl_3$. To eliminate the polysaccharides, an equal volume of water was mixed followed by gentle shaking and allowed to separate the organic phase from the aqueous phase. The lower organic phase containing lipids was collected, evaporated to dryness, quantified and solubilized in 2–3 ml of $CHCl_3$. To analyze the total cell lipids, a diluted fraction was applied on a TLC plate and developed in $CHCl_3$:CH_3OH:H_2O (65:25:4 [vol/vol/vol]). Glycolipids were identified after spraying the TLC plates with 0.2% (wt/vol) anthrone in H_2SO_4 followed by heating, whereas the phospholipids were revealed by the Dittmer reagent [24].

TDM from the total lipid extract containing glycolipids (TDM, TMM) and phospholipids (PI, PIMs, PG/CL) was purified using Quaternary Methyl Ammonium (QMA Spherosil M, Pall BioSepra, Cergy, France) chromatography [23,25]. Before application of the total lipids, the QMA matrix was activated with three times (3×) column volume (CV) of 0.2 M ammonium acetate in $CHCl_3$:CH_3OH (1:2) followed by washing first with 5× CV of $CHCl_3$:CH_3OH (1:2 [vol/vol]) and then with 3× CV of $CHCl_3$. The total lipid mixture in $CHCl_3$ was applied on the activated QMA column up to 100 mg/ml, and a maximum of 200 mg of lipid per 25 g of matrix. The glycolipids were separated by increasing proportions of CH_3OH in $CHCl_3$ such as 10:0, 9:1, 8:2, 7:3, 6:4, 1:1 and 1:2 [vol/vol]. Then the phospholipids were eluted by application of a gradient of ammonium acetate (from 50 to 200 mM) in $CHCl_3$:CH_3OH (1:2 [vol/vol]). Fractions of 10 ml volume were collected and analyzed by TLC. Before quantification, ammonium acetate was removed from the purified phospholipid fractions by re-dissolving the lipid fractions in $CHCl_3$ followed by filtration. For solid state NMR measurements, perdeuterated lipids were obtained from cells grown in D_2O BHI medium. Perdeuterated lipids were extracted and purified similarly as described above. The purity and chemical composition of ^2H labeled or unlabeled TDM and other lipids were analyzed by TLC and MALDI-TOF mass spectrometry.

2.2. Mass spectrometry

MALDI-TOF spectra of purified TDM were acquired on a Voyager-DE STR mass spectrometer (PerSeptive Biosystems). Ionization was achieved by irradiation with a pulsed nitrogen LASER emitting at 337 nm. The spectra were recorded in reflectron mode using an extraction delay of 100 ns and an accelerating voltage of 25 kV. The 2,5-dihydroxybenzoic acid (DHB) matrix (10 mg/ml) was dissolved in $CHCl_3$:CH_3OH (1:1 [vol/vol]). In a typical experiment, 2 μl of TDM $CHCl_3$:CH_3OH 1:1 (1 mg/ml) and 2 μl matrix were mixed and 0.5 μl was disposed onto a metal plate. A total of 2500 shots were accumulated for each sample in a positive ion mode and all data were acquired with default calibration for the instrument.

2.3. Liposome preparation

Three different methods were tried to prepare TDM vesicles:(i) a standard liposome preparation protocol consisting of gentle and direct hydration of a dried lipid film made up of 5 mg of TDM and CL at one of various molar ratios (TDM/CL: 1/0, 4/1, 3/2, 1/1, 2/3 1/4 or 0/1); (ii) a reverse-phase evaporation protocol, as described in [26]; or (iii) a detergent dialysis protocol involving the solubilization of 5 mg of TDM in 2 ml of 10% OG. This TDM–OG nonturbid solution was dialyzed twice against 2 l of water through a 3 kD cut-off membrane with gentle stirring at room temperature, and once at 37 °C. To avoid any organic solvent contamination, all of the lipids were dried overnight in a vacuum pump prior to liposome preparation.