Biophysical properties of membrane lipids of anammox bacteria: I. Ladderane phospholipids form highly organized fluid membranes

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Anammox bacteria that are capable of anaerobically oxidizing ammonium (anammox) with nitrite to nitrogen gas produce unique membrane phospholipids that comprise hydrocarbon chains with three or five linearly condensed cyclobutane rings. To gain insight into the biophysical properties of these ‘ladderane’ lipids, we have isolated a ladderane phosphatidylcholine and a mixed ladderane phosphatidylethanolamine/phosphatidylglycerol lipid fraction and reconstituted these lipids in different membrane environments. Langmuir monolayer experiments demonstrated that the purified ladderane phospholipids form fluid films with a relatively high lipid packing density. Fluid-like behavior was also observed for ladderane lipids in bilayer systems as monitored by cryo-electron microscopy on large unilamellar vesicles (LUVs) and epifluorescence microscopy on giant unilamellar vesicles (GUVs). Analysis of the LUVs by fluorescence depolarization revealed a relatively high acyl chain ordering in the hydrophobic region of the ladderane phospholipids. Micropipette aspiration experiments were applied to study the mechanical properties of ladderane containing lipid bilayers and showed a relatively high apparent area compressibility modulus for ladderane containing GUVs, thereby confirming the fluid and acyl chain ordered characteristics of these lipids. The biophysical findings in this study support the previous postulation that dense membranes in anammox cells protect these microbes against the highly toxic and volatile anammox metabolites.

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1. Introduction

Anammox bacteria play an essential role in the global marine nitrogen cycle and in waste waters as they are capable of anaerobically oxidizing ammonium (anammox) with nitrite into nitrogen gas [1–4]. This naturally occurring process is restricted to a distinct phylogenetic group of Brocadiales that is related to the Planctomycetes that currently holds the five genera Candidatus “Brocadia”, Candidatus “Kuenenia”, Candidatus “Anammoxoglobus”, Candidatus Jettenia, and Candidatus “Scalindua” [3,5–7]. While the 16S rRNA gene sequence similarity of these genera is less than 85%, they possess similar morphological and physiological characteristics [6,8,9]. Their habitats include wastewater treatment systems, where anammox bacteria were originally discovered, as well as fresh water and oceanic systems [1,4,9,10]. A detailed study on the biochemical nature of the anammox process showed that nitrogen gas is produced from equimolar amounts of ammonium and nitrite [11]. While this conversion is the most important energy source for these lithotrophic organisms, it proceeds very slowly and cell proliferation takes place only once every 2–3 weeks [3]. A key enzyme mediating the anammox process is hydroxylamine/hydrazine oxidoreductase [12–14]. Both immunofluorescence microscopy and electron microscopy combined with immunogold labeling revealed that hydrazine oxidase is localized exclusively in a massive intracellular membrane compartment called the anammoxosome [15–18]. In Candidatus Brocadia spp., this organelle-like structure occupies more than 30% of its total cell volume [18]. It has been proposed that the anammoxosomal membrane is of vital importance for preserving an electrochemical proton gradient, which is required for ATP synthesis [18]. Moreover, the intracytoplasmic anammoxosome is also vital for cell viability as it

Abbreviations: 18:1–06:0 NBD-PC, 1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino]hexanoyl]-sn-glycero-3-phosphocholine; SOPs, 1-stearoyl-2-oleoyl-sn-glycero-3-phosphoserine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine; GC–MS, gas chromatography mass spectrometry; GUV, giant unilamellar vesicles; HPLC–ESI–MS/MS, high performance liquid chromatography electrospray ionization tandem mass spectrometry; LUV, large unilamellar vesicle; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SOPs, 1-stearoyl-2-oleoyl-sn-glycero-3-phosphophorylcholine; TLE, total lipid extract; TMA-DPH, 1-[(4-(trimethylamino) phenyl)-6-phenylhexa-1,3,4-triene

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might protect the cell from the highly reactive anammox intermediates hydrazine, nitric oxide and/or hydroxylamine [19]. As these toxins likely can readily diffuse across membranes composed of conventional lipids, the anammoxosome must be comprised of specialized membrane building blocks to provide a dedicated permeability barrier.

Sinninghe Damsté and co-workers have investigated the membrane components of the anammoxosome by GC–MS and high field NMR, and revealed novel lipid structures that are unique in membrane biology. The membranes of anammox bacteria possess for the largest part lipids with alkyl chains possessing three or five cyclobutane rings tandemly fused by cis-ring junctions, designated

![Diagram of lipid structures](image)

**Fig. 1.** Major phospholipids present in anammox bacteria. (A) The general structures of the PC, PE and PG diether lipids are shown by I, III and V, respectively. Structures II, IV and VI depict the ether–ester lipids of PC, PE and PG, respectively. The R1 hydrocarbon chain (a–m) are: (a) C18-[3]-ladderane, (b) C18-[5]-ladderane, (c) C20-[3]-ladderane, (d) C20-[5]-ladderane, (e) C22-[3]-ladderane, (f) C22-[5]-ladderane, (g) pentadecane, (h) 14-methylpentadecane, (i) hexadecane, (j) 9,14-dimethylpentadecane, (k) 10-methylhexadecane, (m) 15-methylhexadecane, with X = COOH or CH3OH. (B) and (C) show the three-dimensional illustration of the [3]- and [5]-ladderane structures, respectively [19].
[3]- and [5]-ladderanes, respectively, with the [3]-ladderanes being condensed to a cyclohexane ring (Fig. 1) [19–21]. The sn-2 position of the glycerol backbone of these lipids is typically occupied by an ether-linked C20-[3]-ladderane moiety [21,22]. The hydrocarbon chains at sn-1 position are more structurally diverse and include a variety of ladderane, straight chain and methyl branched hydrocarbon chains that are either etherified or esterified to the glycerol backbone. The headgroups of these ladderane lipids comprise phosphocholine, phosphoethanolamine or phosphoglycerol moieties [23,24] (Fig. 1). Besides ladderane phospholipids, anammox membranes also comprise the cholesterol-like C135 bacteriophanetetrol [23–25].

Although these extraordinary ladderane lipids have now been structurally well characterized, very little is known about their functional role in the cell. Previously, it was demonstrated that the diffusion of fluorophores across ladderane containing membranes was significantly lower than across conventional membranes [19]. Molecular modeling studies showed that a bilayer composed of a lipid with an etherified C20-[3]-ladderane and an esterified C20-[5]-ladderane hydrocarbon chain formed an exceptionally dense membrane [19].

In the present study, we isolated lipid fractions containing ladderane phosphatidylethanolamine (PE) and ladderane phosphatidylglycerol (PG). We characterized these components in different membrane-like environments such as lipid monolayers and both large and giant phosphatidylethanolamine (PE) and ladderane phosphatidylglycerol (PC) and a mixture of ladderane phospholipids were also examined by micropipette aspiration to elucidate their mechanical properties in GUVs. The results are discussed in the light of lipid packing of highly organized lipid membranes.

2. Materials and methods

2.1. Lipid material

About 5 L of a suspension of anammox biomass grown at 25 °C was collected from an oxygen limited wastewater treatment plant at Paques B.V. (Balk, The Netherlands). Fluorescence in situ hybridization analysis confirmed the dominance of K. stuttgartiensis in the reactor sample as described previously [23]. The suspension was lyophilized yielding ~40 g of dried biomass and the total lipid extract was isolated using a modified Bligh–Dyer procedure [26]. A solvent mixture of phosphate buffer/methanol/dichloromethane 0.8/2.0/1.0 (v/v/v) was added to the biomass. After 10 min of ultrasonication, dichloromethane and phosphate buffer were added to a volume ratio of 0.9/1.0/1.0 (v/v/v), followed by centrifuging to collect the dichloromethane fraction. The residual substance was re-extracted twice using the same procedure and the lipids were harvested from the dichloromethane fraction by rotary evaporation.

For control experiments, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were purchased from Avanti Polar Lipids (Alabaster, AL).

2.2. Analysis of ladderane lipids

For compositional analysis, aliquots of the anammox total lipid extract were dissolved in dichloromethane/methanol ratios 9:1 (v/v) at 1.0 mg mL\(^{-1}\) and subjected to high performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS). For this purpose, an Agilent 1100 series LC (Agilent, San Jose, CA) was used and coupled to a Thermo TSQ Quantum ultra EM triple quadruple mass spectrometer with an Ion Max source equipped with an electrospray ionization probe (Thermo Electron Corporation, Waltham, MA) [23,24]. Liquid chromatography was accomplished on a Lichrospher column (250 mm × 2.1 mm, 5 mm particles; Alltech Associates Inc., Deerfield, IL) maintained at 30 °C. Two linear phase solutions were used: mixture A with hexane/2-propanol/formic acid/14.8 M NH\(_3\) ratios 79/20/0.12/0.04 (v/v/v/v) and mixture B with 2-propanol/water/formic acid/14.8 M NH\(_3\) ratios 88/10/0.12/0.04 (v/v/v/v). The applied flow rate was 0.2 mL min\(^{-1}\), starting with 90% A to 35% A over 45 min, maintained for 20 min, then back flushed to 100% A for 20 min. Lipids were monitored by a data dependent MS routine in positive ion mode. First, a mass scan (m/z 300–1000) was performed. Subsequently, the base peak of the generated mass spectrum was fragmented in a MS/MS experiment [collision energy 20 V, collision gas (argon) 0.8 m Torr]. The [M+H\(^{+}\)] ions of PC lipids were monitored by parent ion scanning for m/z 184, whereas the [M+H\(^{+}\)] ions of PE and the [M+NH\(_{4}\)\(^{+}\)] ions of PG were detected by neutral loss scanning for their characteristic fragments of 141 and 189 Da, respectively.

2.3. Isolation of ladderane phospholipids

Selected phospholipid classes of ladderane lipids were isolated by repeated semi-preparative HPLC using an HP 1100 LC system (Hewlett Packard, Palo Alto, CA) equipped with a thermostatted autoinjector and a column oven coupled to an Isco Foxy Jr. Fraction collector (Lincoln, NE). After injection of the filtered lipid extract onto a LiChrospher DIOL 100 Å (250 × 10 mm, 5 μm; Alltech) column, all fractions were eluted using the identical gradient and mobile phase composition described above for the HPLC-ESI-MS/MS analysis, but with a flow rate of 3 mL min\(^{-1}\). The eluate was collected in 1 min fractions and small aliquots were analyzed by flow injection analysis using the same HPLC system as described above, coupled to an HP 1100 MSD mass spectrometer equipped with ESI source [27]. Fractions containing ladderane PC diether and/or PC ether–ester lipids were pooled and dried by rotary evaporation, yielding a lipid mixture that was designated ‘PC ladderane fraction’. The fractions containing the co-eluting ladderane PE and PG diether and ether–ester lipids were also collected and pooled. The latter is referred to as the ‘PE/PG ladderane fraction’. To remove residual ionic compounds like ammonium formate, both fractions were re-extracted as described above. Subsequently, the purities of all ladderane lipid containing samples were determined by weighing the samples and calculating the inorganic phosphorus contents on the basis of colorimetric measuring [28].

2.4. Langmuir monolayers

Surface pressure–area range per molecule isotherms were obtained using a Teflon\(^{\circledR}\) Langmuir–Blodgett trough with dimensions 20 cm by 30 cm (Type 611, Nima, Coventry, United Kingdom). Lipids were dissolved in chloroform at 1.0 mg mL\(^{-1}\). About 75 μL of a given lipid solution was gently released onto the water subphase at room temperature (23 ± 1 °C) using a 100 μL Hamilton microsyringe (Hamilton, Reno, NV). The barrier speed of the instrument was set at 20 cm\(^{2}\) min\(^{-1}\) while the surface pressure of the film was measured using a Wilhelmy plate made of filter paper. Each surface pressure–area curve was recorded at least three times. Langmuir data was analyzed by determining the isothermal compressibility, \(k_0\), as shown in Eq. (1), where \(A\) and \(\pi\) represent the molecular occupied area and film pressure, respectively:

\[
k_0 = -\frac{1}{A} \frac{dA}{d\pi}
\]

Furthermore, we used the MS data (Fig. 2) to estimate the mean molecular weights of 775 and 750 g mol\(^{-1}\) for the PC and PE/PG ladderane mixtures, respectively.
2.6. Fluorescence microscopy of giant unilamellar vesicles

GUVs were prepared by electroformation as described previously [30–32]. Here, we generated GUVs using PC ladderane lipids with 1 mol% of 1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino] hexanoyl]-sn-glycero-3-phosphocholine (18:1–06:0 NBD-PC) (Avanti Polar Lipids). GUV imaging was performed with Nikon Eclipse 400 fluorescence microscope (Nikon, Melville NY) equipped with a fluorescence filter cube (EF-4 FITC HYQ, Nikon).

2.7. Fluorescence depolarization

Fluorescence depolarization experiments were conducted for the anamox and E. coli total lipid extracts, using a Fluorolog®-3 Instrument (Horiba Jobin Yvon, Edison, NJ), according to Shinitzky and Barenholz [33]. For experiments using 1,6-diphenyl-1,3,5-hexatriene (DPH) (Invitrogen, Eugene, OR), lipids were dried by rotary evaporation and hydrated in 50 mM KP, buffer, pH 7.0 to produce liposomes as described above. LUVs at a lipid concentration of 50 μM in 50 mM KP buffer, pH 7.0 were incubated in the presence of 5.0·10⁻⁶ M DPH. Excitation and emission wavelength were set at 360 nm and 428 nm, respectively. For the 1-[4-(trimethylamino)phenyl]-6-phenylhexa 1,3,4-triene (TMA-DPH) (Invitrogen) labeling of liposomes, 5 μL TMA-DPH stock solution (4 mM in dimethylsulfoxide) was diluted into 245 μL 50 mM KP buffer, pH 7.0. Subsequently, the liposomes were mixed with TMA-DPH solution in a 1:1 volume ratio, resulting in a TMA-DPH/lipid ratio of 1:125 (mol/mol). After incubation for 1 h at room temperature, the steady state fluorescence depolarization was measured upon a 40-fold dilution of the liposomes. The excitation and emission wavelength were recorded at 360 nm and 430 nm, respectively. All experiments were performed using a Lauda Model RE 106 temperature controlled water bath (Lauda-Königshofen, Germany).

2.8. Micropipette aspiration of giant unilamellar vesicles

Mechanical properties of GUVs were studied by video microscopy of micropipette aspiration as described in detail by Longo and Ly [32]. For this purpose, GUVs were prepared as described above, with the exception of adding 0.5 mol% of 1-stearoyl-2-oleoyl-sn-glycero-3-phosphoserine (SOPS) instead of 1.0 mol% 18:1–06:0 NBD-PC to prevent vesicle fusion. Micropipettes with an inner diameter tip of 6 to 8 nm were used after coating with SurfaSil® (Pierce, Rockford, IL). The micropipettes were filled with 100 mM glucose containing 0.02% total wt. bovine serum albumin solution to diminish vesicle adhesion to the glass surface. The position of the micropipette was controlled with a micromanipulator (model MHW-3, Narishige, Japan). Clean GUVs were gently pre-stressed and the aspiration pressure was varied to gradually increase the membrane tension. Alterations in the projection length of the vesicle were recorded on video [32]. We used a Nikon microscope equipped with a 403 Hoffman modulation contrast objective (Modulation Optics, Greenvale, NY), a high-resolution charge-coupled device camera (Dage MTI, Michigan City, IN), a manometer slider with linear encoder (Velmex, Bloomfield, NY) and a video overlay box (Polvision, Western Australia). The suction pressure, ΔP, applied to a vesicle was determined from the change in height of a water manometer coupled to the micropipette. The membrane tension, τ, was calculated using Eq. (2), and depends on the suction pressure and the geometry of both the vesicle and micropipette [32,34].

$$\tau = \frac{\Delta P R_p}{2 \left(1 - \frac{R_p}{R_V}\right)}$$  

The vesicle radius, R_v, and change in length of projection, ΔL, were measured by video imaging and converted into microns through
calibration with a known grid. The radius of the pipette, \( R_p \), was measured by inserting a probe with a known length–diameter relationship, as determined from electron microscopy, into the micropipette. The change in surface area \((A - A_0)\), normalized by the initial area \( A_0 \) equals the area strain, \( \alpha \). The area strain was determined from the projection length using the approximate relationship shown in Eq. (3) [32].

\[
\alpha = \frac{2R_p \Delta L}{A_0} \left( 1 - \frac{R_p}{R_c} \right)
\]

Area compressibility measurements were conducted by gradually increasing the suction pressure and by determining the change in the projection length. The apparent area compressibility modulus, \( K_A \), was obtained from the linear slope of the tension versus area strain in the high-tension regime, given that \( \tau > 0.5 \) mN/m. Similar rates of suction pressure increase were applied during repeated measurements. All experiments were conducted at room temperature.

3. Results and discussion

3.1. Isolation of ladderane phospholipids

Since an axenic culture of anammox bacteria is still lacking to date, we have obtained a total lipid extract from an enrichment (about 75% purity) culture of anammox bacteria derived from a wastewater treatment plant in Balk, the Netherlands. In agreement with previous detailed HPLC-MS/MS and GC–MS characterization studies, most components of the total lipid extract were PC, PE and PC lipids that possess at least one ladderane hydrocarbon chain (see Fig. 1 and [21–24]).

For the study of biophysical properties of ladderane lipids it would be desirable to obtain individual ladderane phospholipid species in high purity. Unfortunately, the chromatographic separation of these lipids is mainly determined by polarity of their headgroup moiety rather than the nature of the hydrocarbons [23,24,35]. Furthermore, due to the large molecular diversity in ladderane lipids, it would have been difficult to obtain purified material in sufficient quantities. We therefore isolated a fraction containing only PC diethers/ether–ester ladderane lipids and a fraction containing the PE and PG diethers/ether–ester ladderane lipids (purity about 85%). Fig. 2 depicts the ESI-MS/MS spectra for the isolated PC- and PE/PG ladderane fractions. These profiles showed that they exclusively consist of phospholipids with one or two ladderane hydrocarbon chains.

3.2. Packing of ladderane phospholipid monolayers

We performed Langmuir monolayer experiments at room temperature \((23 \pm 1 ^\circ \text{C})\) to obtain information on the biophysical behavior of ladderane lipids, using DSPC and SOPC as references. After the lift-off, the solid phase DSPC monolayer exhibited an onset pressure of ladderane lipids, using DSPC and SOPC as references. After the lift-off at a temperature (23±1°C) to obtain information on the biophysical behavior of the ladderane phospholipid monolayers with one or two ladderane hydrocarbon chains.

3.3. Formation of large and giant unilamellar vesicles

The proposed dense and fluid ladderane lipid architecture was further investigated in a lipid bilayer environment using LUVs. Imaging of these vesicles by cryo-electron-microscopy showed that both PC- and PE/PG ladderane containing LUVs were spherically shaped at room temperature, hence characteristic for vesicles comprising fluid-phase lipids (Fig. 4A and B, cf. [29]). We estimated that the ladderane liposomes exhibit a membrane thickness near 4 nm, which is similar to that of membranes comprised of DPPC [39]. A ChemProMM2 calculation of one of the most abundant PC ladderane lipid (Fig. 1B) in minimal energy configuration suggest a length of ~2.4 nm for this lipid and thus a bilayer composed of this membrane would have a thickness of maximally ~4.8 nm. The slightly lower observed bilayer thickness suggests there may be some interdigitation of the ladderane phospholipids bilayers.
GUVs are useful model systems of biological membranes to investigate lipid domain formation and phase behavior at macroscopic scales, as well as membrane deformation processes and mobility of membrane components [31,40,41]. Fig. 4C shows a typical confocal image of GUVs composed of the isolated PC ladderane lipid mixture labeled with 18:1–06:0-NBD-PC at room temperature. Since the probe preferentially co-localizes with fluid-phase lipids, the homogeneous fluorescence intensity distribution of the spherical PC ladderane GUVs points toward the existence of a single fluid phase, at least on the macromolecular scale probed by light microscopy. The scale bar represents 25 μm.

Fig. 4. Microscopy analysis of ladderane lipid vesicles. Cryo-electron images of LUVs composed of (A) the PC ladderane fraction and (B) the PE/PG ladderane fraction. The scale bars indicate 100 nm. (C) GUVs composed of PC ladderane lipids labeled with 1 mol% of 18:1–06:0 NBD-PC showing a homogenous fluorescence corresponding to a single fluid phase, at least on the macromolecular scale probed by light microscopy. The scale bar represents 25 μm.

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3.4. Acyl chain ordering in ladderane phospholipids

The acyl chain ordering of the ladderane lipid containing membranes was examined by using the fluorescent membrane-embedded probes DPH and TMA-DPH [33,44,45]. Fig. 5A shows the fluorescence intensities for DPH which preferentially anchors within the interior of the hydrophobic region of bilayers of LUVs [46]. The reference DPPC LUVs showed DPH fluorescence depolarization intensities higher than 0.44 at temperatures below its phase transition at 42 °C (Fig. 5A, grey circles). In agreement with earlier studies, lower fluorescence depolarization intensities were observed at elevated temperatures due to more rotational freedom of the probe [47–49]. For the PC ladderane fraction, the temperature dependent DPH fluorescence depolarization profile was significantly different (Fig. 5A, black circles). In the absence of a phase transition, the DPH fluorescence intensity almost linearly decreased from about 0.40 to 0.14. At room temperature, the DPH fluorescence intensity of the PC ladderane fraction was close to 0.26, indicating fluid-like membranes.

Fig. 5. (A) Fluorescence depolarization of DPH in membrane vesicles prepared from DPPC (gray circles), the PC ladderane fraction (black circles) and the PE/PG ladderane fraction (white circles). (B) Fluorescence depolarization of TMA-DPH in membrane vesicles from DPPC (gray circles), the PC ladderane fraction (black circles) and the PE/PG ladderane fraction (white circles). The data points of the latter two represent the mean values ± the standard deviation of three independent experiments.
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