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A NEW TYPE OF CELL MEMBRANE, IN THERMOPHILIC ARCHAEBACTERIA, BASED ON BIPOLAR ETHER LIPIDS*

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Summary

The complex membrane lipids of thermophilic archaebacteria are bipolar amphipathic molecules. Indirect evidence suggests that the bipolar lipids of the membrane of these microorganisms have a monolayer organization, similar to a "unit membrane" bilayer, with molecular elements, the covalent binding of which spans its entire thickness.

We report further evidence that the membrane bipolar lipids of *Caldariella acidophila*, an extreme thermophilic archaebacterium, can be organized as a covalently bound bilayer, in which each molecule, fully stretched, anchors the two polar heads to the inner and outer faces, respectively, of the membrane array.

Introduction

Archaebacteria, the third primary kingdom of cells in addition to eukaryotes and eubacteria [1—2], are all characterized by unusual isoprenoid ether lipids [3—13]. In particular, the complex membrane lipids of thermophilic archaebacteria [8—13] are bipolar amphipathic molecules which, irrespective of complex lipid structures, have two polar heads linked together by C_{40} isoprenoid chains (Fig. 1), that differ from each other by the feature of up to four cyclopentane rings [13]. This unusual molecular architecture gives rise to substantial modifications in the organization of the lipidic layer of the membrane, which still retains the typical "trilaminar" structure [14—16] shown by electron micrographs of section of all biological membranes.

A series of indirect evidence such as the structure and dimension of lipids themselves [10, 11], the absence of a preferential fracture plane on the middle of the lipid layer [17], the extreme rigidity of the thermophilic archaebacteria membrane [17] and some properties of black lipid membranes from lipids of *Caldariella acidophila* [18], an extremely thermoacidophilic archaebacterium [14, 19], suggest that the membrane lipids of these microorganisms have a monolayer organization which is similar to a "unit membrane" bilayer,

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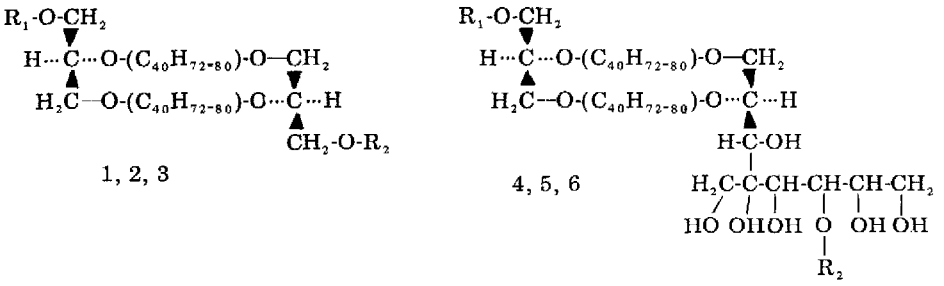

Compound		R_1	R_2	(%)
1	GLI	H	β-D-gal*p*←β-D-glc*p*	5.8
2	PLI	P-inositol	H	7.8
3	PGLI	P-inositol	β-D-gal*p*←β-D-glc*p*	7.8
4	GLII	H	β-D-glc*p*	13.6
5	SGLII	H	β-D-glc*p*-sulphate	10.4
6	PGLII	P-inositol	β-D-glc*p*	54.5

Fig. 1. Structures of complex membrane lipids of *C. acidophila* and their relative percentages in optimal growth conditions (87°C, pH 3.5) [12].

with molecular elements, the covalent binding of which spans its entire thickness. We have obtained further evidence that, in vivo, the membrane bipolar lipids of thermophilic archaebacteria can be organized as a "true monolayer", in which each molecule is simultaneously attached to the inner and outer faces of the membrane array.

Materials and methods

Chemicals

Glycosidase mixture from *Charonia lampas*, purchased from Seikagaku Kagyo, Tokyo, contains the following active enzymes: α- and β-mannosidase, α- and β-glucosidase, α- and β-galactosidase, α-L-fucosidase, β-xylosidase, α- and β-*N*-acetylglucosaminidase and α- and β-acetylgalactosaminidase. [2-^{14}C] mevalonate and [^{35}S] sodium sulphate were purchased from Radiochemical Centre, Amersham (Gt. Britain). Silica gel TLC plates were from Merck, Darmstadt. Diaflo ultrafiltration membranes were from the Amicon Corporation. All other chemicals were from local sources and of the best grade available.

Cell culture and lipid extraction

Caldariella acidophila, strain MT-4, was isolated from a hot acidic spring in Agnano, Naples. The bacterium was grown as in [19], by adding to a 300 ml culture medium either 50 μCi of [2-^{14}C] mevalonate or 0.5 mCi of [^{35}S]-sodium sulphate; in the latter case the growth medium was modified to

avoid dilution of isotope. Cells were harvested in the late stationary growth phase by centrifugation at 33,000 *g*. The wet labelled cells were mixed with 12 g of wet unlabelled cells, grown in the same conditions. Lipids were extracted from cells according to Kates [20].

Characterization of hydrolysis products of C. acidophila *lipids treated with glycosidases from* C. lampas

5 g of complex lipids of *C. acidophila* were resolved as reported in Ref. [12] and 100 mg of different lipid fractions, dissolved by sonication in 5 ml of acetate buffer of pH 4.5, were incubated at 40°C for 72 hr with 50 mg of glycosidases from *C. lampas*. The reaction mixture was extracted with $CHCl_3$:MeOH (1:1 v/v) and the hydrolysis products, soluble in $CHCl_3$, were purified on a silica gel column, as reported in [12], and identified chromatographically and spectroscopically.

Glycosidase fractionation

Crude glycosidases from *C. lampas* were fractionated by ultrafiltration in a micro-Amicon cell, using an XM 50 membrane with a cut-off of 50,000 to remove low molecular weight material. The high molecular weight fraction was then lyophilized and used for lipid hydrolysis.

Glycosidase digestion: hydrolysis of lipids localized in the membrane of intact cells

12 g of *C. acidophila* cells, grown in the presence of [2-^{14}C] mevalonate were shaken in 50 ml of acetate buffer of pH 4.5 at 40°C with 250 mg of glycosidase mixture from *C. lampas*, adding two aliquots of 100 mg of the enzymes at 24 and 72 hr. At different times, 8 ml aliquots of the suspension were separately centrifuged at 10,000 *g* for 30 min. The cells were then extracted and the lipids chromatographed. A control experiment without the addition of glycosidases was performed in the same way.

Glycosidase digestion: hydrolysis of lipids as free molecules

Radioactive lipids, obtained from 12 g of cells of *C. acidophila*, grown in the presence of [2-^{14}C] mevalonate or [^{35}S] sodium sulphate, were dissolved by sonication in 25 ml of acetate buffer of pH 4.5 and incubated at 40°C with 150 mg of glycosidase mixture from *C. lampas*, adding two aliquots of 50 mg of the enzymes at 24 and 48 hr. 5 ml of the reaction mixture were withdrawn at different times and extracted with $CHCl_3$:MeOH (1:1 v/v). The lipid extract was then analyzed by chromatography. A control experiment, without the addition of glycosidases, was performed in the same way.

Chromatographic procedures

The lipid extracts were resolved on TLC plates, developed with $CHCl_3$: MeOH:H_2O (65:25:4 by vol.) or with $CHCl_3$:Et_2O (95:5 v/v). The spots, revealed by I_2 vapour, were scraped and the radioactivity, associated with the different compounds, was measured by liquid scintillation.

Methylthioadenosine phosphorylase assay

The enzyme activity, determined as in Ref. [21], was evaluated at the end of the incubation experiments of *C. acidophila* cells with *C. lampas* glycosidases, in the incubation media and in the incubated cell homogenate [21].

Electron microscopy

Intact and glycosidase treated cells were analyzed by electron microscopy as reported in [14].

Results and discussion

The membrane lipids of the extremely thermoacidophilic microorganism *C. acidophila*, which grows optimally at 87°C [14, 19], are based on macrocyclic tetraethers (Fig. 1, R_1=R_2=H), designated "glycerol-dialkyl-glycerol tetraethers" [10], and "glycerol-dialkyl-calditol tetraethers" in which one glycerol unit is substituted by a branched chain nonitol, called "calditol" [11].

All the complex lipids of this thermophilic archaebacterium derive from these tetraethers, to the free hydroxyls of which other molecular groups, such as sugar and/or inositol phosphate, are linked (Fig. 1). These lipids, previously partially characterized [12], have been more extensively defined on the basis of ^{13}C NMR spectroscopy (data not given), that has localized the inositol phosphate group on the glycerol, and the glucose on the calditol skeleton. In optimal growth conditions [12], lipids with glycosidic linkages on one of the polar heads constitute 92% of the total complex lipids (Fig. 1).

Isoprenoid ether lipids are almost exclusively localized on the cell membrane. In fact, the acidic hydrolysis [19] of the cell wall fraction, prepared according to Weiss [22], afforded macrocyclic tetraethers (Fig. 1, R_1=R_2=H) in small amount (~ 2–3%), where 100% refers to the total tetraethers recovered by acidic treatment of cell envelopes (cell membrane plus cell wall).

Our aim was to determine the amount of lipid heads with glycosidic linkages exposed on the external surface of the cell membrane. The approach was to treat intact cells of *C. acidophila* with a non penetrating reactant which was able to permeate the cell wall and to hydrolyze the glycosidic bonds of the membrane lipids of the microorganism. The reactant used was a mixture of glycosidases obtained from the marine gastropod *C. lampas*. These enzymes completely hydrolyzed all the glycosidic linkages of purified membrane lipids of *C. acidophila* in about 72 hr, giving rise to the molecular species of Fig. 1 in which R_2=H.

The use of *C. lampas* glycosidases to localize the lipid polar heads with glycosidic bonds on the external surface of the cell membrane of *C. acidophila* presupposes a series of important conditions: (a) the reactant must never enter the cell; (b) it must be able to hydrolyze all the glycosidic linkages on the external face of the membrane; and (c) the cell membrane must

not be damaged during the reaction. Our experimental conditions satisfy all these requirements. In fact, *C. lampas* glycosidases (m.w. higher than 50,000) are non penetrating reactants [23]; moreover the mild experimental conditions used in the procedures described above, even after 120 hr of glycosidase treatment, do not cause cell lysis or modifications in the morphology of the membrane, as shown by electron micrographs of cell sections. Further evidence of cell integrity and of membrane impermeability to proteic macromolecules during the glycosidase digestion, is the absence in the incubation medium of cytoplasmatic enzymes such as methylthioadenosine phosphorylase (MTA phosphorylase E.C. 2.4.1.1.), a highly thermostable enzyme of *C. acidophila* (m.w. 90,000) [21] that remains localized in the cytosol after incubation with *C. lampas* glycosidases. No MTA phosphorylase activity can be detected in the incubation medium, while in the homogenate of treated cells the amount of MTA phosphorylase is comparable to that assayed in a control of cells not treated with glycosidases.

We performed the glycosidase digestion experiments with cells of *C. acidophila* labelled with either [2-^{14}C]mevalonate or [^{35}S]sulphate. The first precursor specifically labels the isoprenoid component of all lipids [23], while [^{35}S]sulphate is incorporated only in the sulphoglycolipids SGLII (Fig. 1).

The analysis of radioactive lipids, extracted from [2-^{14}C]mevalonate cells of *C. acidophila* incubated for different times with glycosidases from *C. lampas* shows that after 120 hr, glycolipids GLI and GLII and phosphoglycolipids PGLI and PGLII were completely hydrolyzed, while the sulphoglycolipids SGLII remained unmodified (Table 1).

TABLE 1

Hydrolysis of glycosidic linkages of *C. acidophila* lipids labelled with [2-^{14}C]mevalonate* localized in the membrane of intact cells (a)†§ and as free molecules† (b)

Incubation time (hr)	24		48		72		96	120
Sample nature / Lipid+	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(a)
	Hydrolysis (%)							
GLI	18	76	42	89	51	100	90	100
PLI	0	0	0	0	0	0	0	0
PGLI	34	74	42	86	70	100	89	100
GLII	20	77	40	87	45	100	84	100
SGLII	0	71	0	84	0	99	0	0
PGLII	36	75	41	88	64	99	76	100

*The different lipids, specifically labelled on the isoprenoidic chains, have the same molar radioactivity [24].

†For experimental conditions see Material and Methods.

+For structures see Fig. 1.

§*C. acidophila* cells, incubated at pH 4.5, 40°C for 120 hr, without glycosidases, do not show degradation of membrane lipids.

A parallel experiment performed with [^{35}S]*sulphate cells of C. acidophila* specifically confirmed that sulphoglycolipids SGLII are not hydrolyzed when inserted into membrane, and that they are completely degraded from *C. lampas* glycosidases as free lipids.

Two possible models can be considered for amphipathic molecules: a monolayer organization of the bipolar lipids, in which each molecule is simultaneously attached to the inner and outer faces of the membrane array, or two opposed monolayers in which both polar heads of each molecule are anchored to the same face of the membrane array, inducing a folding of the isoprenoidic chains (this may be called the U-shaped configuration).

Our results indicate that at least 82% of total lipids have a polar head exposed outside the membrane array. This experimental evidence, according to the hypothesis of a double layer of lipids in the U-shaped configuration, requires that at least 82% of total complex lipids be localized on the outer layer, whilst only 18% should be, at maximum, localized in the cytoplasmatic lipid layer. Considering that the average values of the area/molecule ratio of molecules localized on two lipid layers are about the same, it could be hypothesized that the mass deficiency in the inner lipid layer might be compensated by proteins occupying a greater area of the inner face than of the outer face.

Such a type of membrane model, for about 78%, could be a monolayer of U-shaped bipolar lipids that expose the two polar heads outside and the aliphatic component inside. Experimental evidence obtained with electron microscopy (thickness of lipid layer [14] and freeze-etching experiments [17]) and well known principles that justify a double layer organization of conventional lipids in biological membranes, are not in agreement with this hypothesis. In contrast, our results support well the hypothesis of a monolayer organization of lipids in which the molecules, when fully stretched, span their thickness by anchoring the two polar heads to the inner and outer faces, respectively, of the membrane array.

Our experimental results also show that the membrane of *C. acidophila* is one of the most regular structures of this type known so far; in fact, the complete hydrolysis of glycolipids GLI and of phosphoglycolipids PGLI and PGLII (82% of complex membrane lipids) implies that all these molecules have their polar heads with glycosidic linkages exposed outside.

At present, nothing may be said concerning the orientation of the remaining 18% of lipids, which are represented by phospholipids PLI without glycosidic linkages, and sulphoglycolipids SGLII that are inaccessible to the enzymatic action when inserted into the intact cellular membrane.

The structural asymmetry of the complex lipids of *C. acidophila* and their regular disposition in the lipid layer means that the membrane of this microorganism always has a polarity, even in the absence of any associated proteins.

This new type of biological membrane presents interesting problems concerning the insertion of neosynthesized lipids in the monolayer and in the topography of membrane proteins.

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