Experimental report

09/02/2016

Proposal: 8-02-746 **Council:** 4/2015

Title: Dependence of Amphotericin B activity in Pichia Pastoris yeast on membrane sterol content

Research area: Biology

This proposal is a new proposal

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Samples: ergosterol
Silicon blocks
Pichia pastoris total lipids (hydrogenous)
deuterated Pichia Pastoris total lipids
deuterated ergosterol

Instrument	Requested days	Allocated days	From	To
D17	4	0		
FIGARO	4	4	26/10/2015	30/10/2015

Abstract:

We have recently carried out a detailed characterization of the lipid composition and structure of native fungal membrane extracts from Pichia Pastoris, as well as the structural consequences of the antifungal agent AmB action on yeast phospholipid membranes. The structure of yeast phospholipid membranes differs considerably from typical model lipid bilayers composed of synthetic lipids and depends on the degree of lipid polyunsaturation as well as the presence of the native ergosterol. AmB inserts in yeast membranes both in the absence and presence of ergosterol, and forms a thick extra-membraneous aggregate in agreement with a recently proposed new model in which it acts as an ergosterol-extracting sponge. We propose to continue the previous investigation by using total lipid extracts from P. pastoris, which also include the other non-polar lipids (diglycerides, sterol esters), and will investigate the effect of membrane sterol content on the mechanism of AmB

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FIGARO 8-02-746

Abstract:

We have recently carried out a detailed characterization of the lipid composition and structure of native fungal membrane extracts from Pichia Pastoris[1], as well as the structural consequences of the antifungal agent AmB action on yeast phospholipid membranes[2]. The structure of yeast phospholipid membranes differs considerably from typical model lipid bilayers composed of synthetic lipids and depends on the degree of lipid polyunsaturation as well as the presence of the native ergosterol. AmB inserts in yeast membranes both in the absence and presence of ergosterol, and forms a thick extra-membraneous aggregate in agreement with a recently proposed new model in which it acts as an ergosterol-extracting sponge[3]. We propose to continue the previous investigation by using total lipid extracts from P. pastoris, which also include the other non-polar lipids (diglycerides, sterol esters), and will investigate the effect of membrane sterol content on the mechanism of AmB. We also want to quantify ergosterol extraction by using membranes where the lipids and ergosterol are selectively labelled in turn as well as investigate the ability of AmB to extract cholesterol found in mammalian membranes, to elucidate the mechanism of AmB's toxic side effects.

Background:

Physiologically, natural yeast membranes are functional at the growth temperature (in our case 30°C) and are composed of around 15-30mol% of ergosterol. The viability of the cells depends on it's membrane composition, and the strong antifungal agent amphotericin B (AmB) is known to interact with the ergosterol and induce fungal or protozoal cell death. Moreover, due to it's preference to ergosterol, mammalian cells containing cholesterol as main sterol are less impacted, explaining the use of AmB as antifungal treatment. In our experiments and studies, we perform deposition of bilayers by vesicle fusion deposition, allowing us to use different compositions in order to investigate the influence of sterol content in the mechanism of AmB. Most of the studies are performed with simple model membranes, composed of one phospholipid molecular specie and sterol content, when we use a natural total lipid extract or a mixture of phospholipids, hydrogenated and deuterated, to which we add sterol content. This experiment aimed at investigating the extraction of ergosterol by AmB and in the same time, the mechanism inducing toxic side effect, by interaction with cholesterol.

Results:

We fully measured 7 different samples during the four days allocated on Figaro. The mixtures were first sonicated in D_2O or H_2O containing 100mM of NaCl and 20mM of $CaCl_2$, before being injected in the sample cells at a controlled 52°C temperature. Some depositions went wrong, notably some of the samples, mainly deuterated lipid in H_2O tended to form partial double bilayers when the silica-water interface is located below the solution in the reflection down geometry of FIGARO, and the more dense deuterated vesicles sediment to the bottom of the sample cell. Those partial double bilayers (stacks) could not be removed by rinsing or osmotic shock treatment and thus the interpretation of the AmB results in those samples is difficult. Nevertheless, we eventually figured out that the double bilayer formation could be prevented by using a solvent (H_2O or D_2O) with the same deuteration as the lipids..The samples are kept in the cells for half an hour before rinsing with pure solvent and cool to 30°C, temperature of measurements. We measured 3 to 4 contrasts (D_2O, CmSi, CM4 and H_2O) before AmB injection for each sample and 3 contrasts (D_2O, CM4 and H_2O) after AmB injection, in order to get the maximum information about the complex lipid-AmB system.

Samples (all 30mol%)	FIG 8-02-746	Contrasts before	Contrasts after
hPolar hErg	✔	4	3
dPolar dErg	Partial double bilayer	3	3
hPolar dErg	Very low deposition	3	3
hPolar hChol	✔	3	3
dPolar hChol	Good but 717 better	4	3
dPolar hErg	Partial double bilayer	3	3

*Pol stands for Polar fraction (phospholipids), Erg for ergosterol and Chol for cholesterol. h & d are the indication of hydrogen or deuterium-labelled molecules.

The main samples we can compare for the moment are the fully hydrogenated Polar-(30mol%) ergosterol and Polar-(30mol%) cholesterol. The dPolar-(30mol%) cholesterol being performed during the previous experiment confirm the changes observed in the hydrogenated corresponding one.

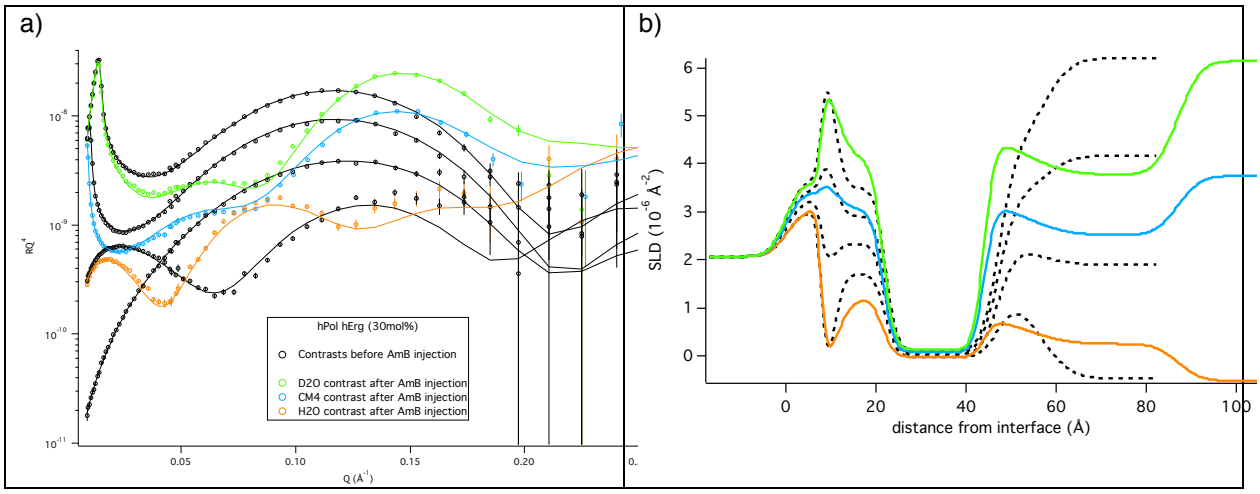

Figure 1, a) Reflectivity profiles of reconstituted hydrogenad polar lipid + 30mol% hydrogenated ergosterol in 4 contrasts before AmB injection: D_2O, CmSi, CM4 and H_2O (black) and 3 contrasts after AmB injection: D_2O (green) CM4 (blue) and H_2O (orange). b) Corresponding SLD profiles.

hPol hErg (30mol%)	Thickness (Å)	Hydratation (%)	roughness (Å)
Head	11.2	20.1	1.8
Chains	25.0	2	2
Head	9.5	37.8	3
hPol hErg (30mol%)	**Thickness (Å)**	**Hydratation (%)**	**roughness (Å)**
Head	10.6	38.1	3
Chains	22.5	0.7	2
Head	9.8	52.3	3
AmB layer	33.3	47.4	7

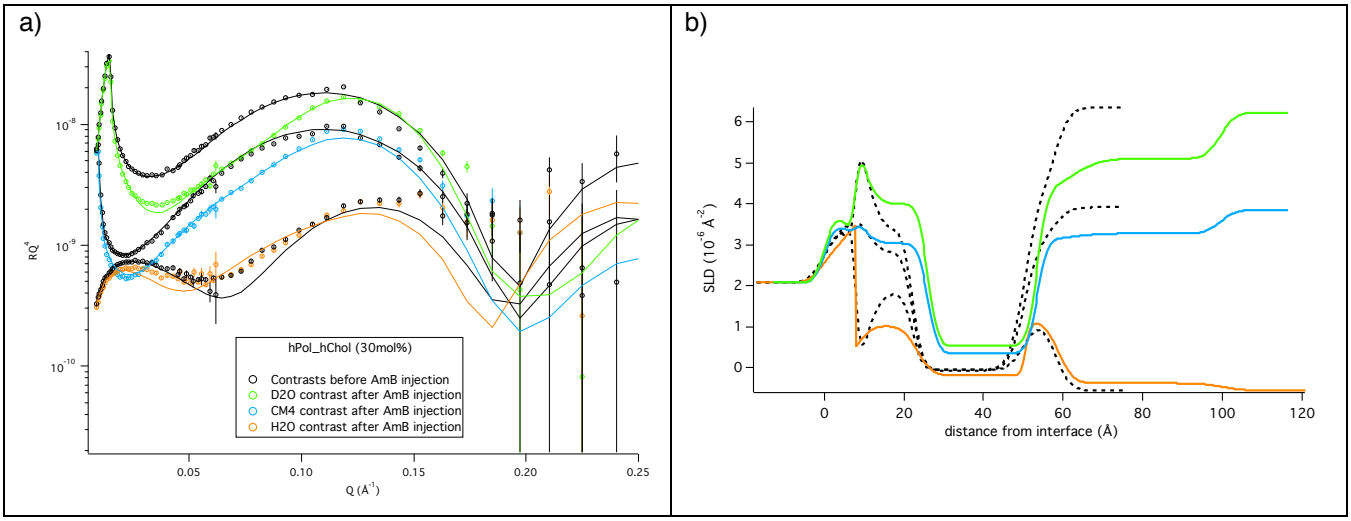

Figure 2, a) Reflectivity profiles of reconstituted hydrogenated polar lipid + 30mol% hydrogenated cholesterol in 3 contrasts before AmB injection: D_2O, CM4 and H_2O (black) and 3 contrasts after AmB injection: D_2O (green) CM4 (blue) and H_2O (orange). b) Corresponding SLD profiles.

hPol hChol (30mol%)	Thickness (Å)	Hydratation (%)	roughness (Å)
Head	11.9	15.5	3
Chains	28.0	0.2	2
Head	7.9	38.2	3
hPol hChol (30mol%)	**Thickness (Å)**	**Hydratation (%)**	**roughness (Å)**
Head	10.6	38.4	3
Chains	26.9	0	2
Head	8.2	48.5	2
AmB layer	41.9	89.4	6

The differences between ergosterol and cholesterol containing membranes are small – in the fitting uncertainty range - before AmB (see structural parameters above). After addition of AmB the membranes "react" in the same way: tails are shorted (2-3Å) when heads are getting more hydrated (+15-25%). The main changes are observed for the AmB layer, for cholesterol containing membranes, an upper AmB layer (around 40Å) with high dilution (89% solvent) is observed. (37Å – 68% hydration for dPol-30mol% cholesterol) with a volume ratio 60/40 of AmB/Cholesterol in both cases. On the opposite, ergosterol containing membranes have a thinner AmB upper layer (35Å) with 50% of solvent and around 50% ergosterol/50%AmB.

References:

1. Production and analysis of perdeuterated lipids from Pichia pastoris cells, de Ghellinck A., Schaller H., Laux V., Haertlein M., Sferrazza M., Marechal E., Wacklin H., Jouhet J., Fragneto G., PloS One, (2014), 9, e92999-1-e92999-9
2. Lipid polyunsaturation determines the extent of membrane structural changes induced by Amphotericin B in *Pichia pastoris* yeast, de Ghellinck A, Fragneto G, Laux V, Haertlein M, Jouhet J, Sferrazza M, Wacklin H. Biochim Biophys Acta. 9, 2317-2325 (2015)
3. Anderson, T.M. et al. Amphotericin forms an extramembranous and fungicidal sterol sponge. Nat Chem Biol 10, 400-406 (2014).