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Lipid topology and physical properties of the outer mitochondrial membrane of the yeast, *Saccharomyces cerevisiae*

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The outer membrane of yeast mitochondria was studied with respect to its lipid composition, phospholipid topology and membrane fluidity. This membrane is characterized by a high phospholipid to protein ratio (1.20). Like other yeast cellular membranes the outer mitochondrial membrane contains predominantly phosphatidylcholine (44% of total phospholipids), phosphatidylethanolamine (34%) and phosphatidylinositol (14%). Cardiolipin, the characteristic phospholipid of the inner mitochondrial membrane (13% of total phospholipids) is present in the outer membrane only to a moderate extent (5%). The ergosterol to phospholipid ratio is higher in the inner (7.0 wt.%) as compared to the outer membrane (2.1 wt.%). Attempts to study phospholipid asymmetry by selective degradation of phospholipids of the outer leaflet of the outer mitochondrial membrane failed, because isolated right-side-out vesicles of this membrane became leaky upon treatment with phospholipases. Selective removal of phospholipids of the outer leaflet with the aid of phospholipid transfer proteins and chemical modification with trinitrobenzenesulfonic acid on the other hand, gave satisfactory results. Phosphatidylcholine and phosphatidylinositol are more or less evenly distributed between the two sides of the outer mitochondrial membrane, whereas the majority of phosphatidylethanolamine is oriented towards the intermembrane space. The fluidity of mitochondrial membranes was determined by measuring fluorescence anisotropy using diphenylhexatriene (DPH) as a probe. The lower anisotropy of DPH in the outer as compared to the inner membrane, which is an indication for an increased lipid mobility in the outer membrane, was attributed to the higher phospholipid to protein and the lower ergosterol to phospholipid ratio. The data presented here show, that the outer mitochondrial membrane, in spite of its close contact to the inner membrane, is distinct not only with respect to its protein pattern, but also with respect to its lipid composition and physical membrane properties.

Introduction

Mitochondria contain two membranous fractions, the inner and the outer mitochondrial membrane. Between these two membranes the soluble intermembrane space is entrapped, but membrane linkage via contact sites ('junctions') has been observed [1]. Nevertheless the outer and the inner membrane are different with respect to their chemical composition and biological function.

The outer mitochondrial membrane is the site of interaction of mitochondria with the cytosol

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and with other organelles. Very little is known about the function of this membrane. Recently we were able to demonstrate that the outer mitochondrial membrane of yeast is one major cellular site of phospholipid biosynthesis [2]. This membrane is also a barrier to be crossed by phospholipids transported out of and into mitochondria. Phosphatidylethanolamine, which is synthesized via decarboxylation in the inner mitochondrial membrane, must be transported to microsomes, where it is required as a substrate for phosphatidylcholine synthesis. Phosphatidylcholine, a major component of the inner mitochondrial membrane, must be imported from the endoplasmic reticulum, where it is synthesized [3]. Therefore, the outer mitochondrial membrane represents a suitable object to study the as yet poorly understood intracellular transmembrane transport of phospholipids. A prerequisite to study this phenomenon is the knowledge of the topology of membrane phospholipids and of the physical properties of the membrane. Such studies of the outer membrane of mitochondria have not been reported before. The outer membrane of yeast mitochondria can be isolated largely free of contaminations with other cellular membranes [4]. A further advantage is the fact that isolated vesicles of the outer membrane are sealed and 'right-side-out' oriented [5].

Lipid analyses of mitochondrial membranes from mammalian and plant cells (for a review, see Ref. 6) showed that the outer and the inner mitochondrial membrane can be distinguished mainly by two facts: the outer membrane contains markedly less cardiolipin, but more sterols than the inner membrane; the phospholipid to protein ratio is significantly higher in the outer as compared to the inner membrane. Similar results were obtained with the microorganism *Neurospora crassa* [7]. Also in yeast cells [8] the phospholipid to protein ratio is higher in the outer than in the inner mitochondrial membrane, but the sterol to phospholipid ratio was found to be rather similar in both membranes [9]. Bednarz-Prashad and Mize [8] studied the phospholipid composition of yeast mitochondrial membranes in an inositol-deficient strain of *Saccharomyces uvarum* (formerly *Saccharomyces carlsbergensis*). They observed a marked difference of the cardiolipin content between the outer and the inner membrane, although their membrane separation procedure did not allow a proper isolation of the outer mitochondrial membrane as judged by the protein pattern and by marker enzyme measurements.

In this paper we present a detailed analysis of the lipids of the outer mitochondrial membrane from a wild-type yeast, *Saccharomyces cerevisiae*. For the first time we show the transbilayer distribution of phospholipids within this membrane. Membrane fluidity of the outer mitochondrial membrane was measured by fluorescence spectroscopy and compared to that of other cellular membranes.

Materials and Methods

Culture conditions and subcellular fractionation

The wild-type yeast strain *Saccharomyces cerevisiae* D 273-10 B was grown aerobically at 30 °C on 2% lactate [4]. Preparations of spheroplasts, mitochondria and microsomes have been described [4]. Submitochondrial fractionation was carried out by published procedures [4,5].

Cross-contamination of fractions was measured using the marker enzymes kynurenine hydroxylase (outer mitochondrial membrane) [10], succinate dehydrogenase (inner mitochondrial membrane) [11] and NADPH–cytochrome-*c* reductase (microsomal fraction) [12], or by immunotitration [13] using monospecific antisera against porin (outer mitochondrial membrane), ADP/ATP translocator (inner mitochondrial membrane) and 40 kDa protein (microsomal fraction) [5]. Subcellular and submitochondrial fractions were checked routinely by SDS-polyacrylamide gel electrophoresis [14]. Protein bands stained with Coomassie blue were scanned on a Shimadzu CS-930 scanner and quantitated by integration.

Preparation of radioactively labelled outer mitochondrial membrane

Saccharomyces cerevisiae D 273-10 B (8 liter culture medium) was grown over night in the presence of 300 μCi [^{3}H]oleic acid (10 Ci/mmol), 30 μCi [^{3}H]inositol (20 Ci/mmol) or 50 μCi [^{3}H]choline (80 Ci/mmol), respectively. Subcellular and submitochondrial fractions were isolated as described above yielding preparations of the outer mitochondrial membrane with the following

specific radioactivities: 250 nCi [^{3}H]oleic acid/mg protein; 3.8 nCi [^{3}H]inositol/mg protein; 4.9 nCi [^{3}H]choline/mg protein.

Asymmetry studies using phospholipid transfer proteins

Enriched fractions of phosphatidylcholine/phosphatidylinositol transfer proteins from bovine heart [15] and from yeast [16] were isolated by published procedures. Non-specific lipid transfer protein from bovine liver [17] was a gift from J. Op den Kamp, Utrecht, The Netherlands.

Incubations with outer membrane vesicles were carried out as follows. Radioactively labelled outer membrane vesicles (70–80 μg phospholipid/ml) were suspended in 10 mM Tris-HCl (pH 7.4). Phospholipid transfer proteins or transfer protein-enriched fractions, respectively, were added to a final concentration of 200–250 U/ml. One transfer unit (1 U) is defined as 1% phospholipid transferred in a standard assay [16]. Incubations with the bovine heart or the bovine liver transfer protein were carried out in the presence of 10 mM mercaptoethanol. Transfer assays were started by the addition of yeast mitochondria (12000*g* pellet) as acceptor membranes. Phospholipids in the acceptor vesicles were present in a 30-fold excess (approximately 2.25 mg phospholipid/ml) over those in the donor vesicles. At time points indicated aliquots were withdrawn and donor and acceptor vesicles were separated in a microcentrifuge (3 min at 12000 × *g*). Lipids from the pellet and the supernatant were extracted with chloroform/methanol (2:1, v/v). After the addition of unlabelled yeast lipids as a carrier individual lipids were separated by two-dimensional thin-layer chromatography (see below) and counted for radioactivity.

Asymmetry studies using 2,4,6-trinitrobenzenesulfonic acid (TNBS)

Outer mitochondrial membrane vesicles labelled with [^{3}H]oleic acid (see above) were suspended in an incubation buffer containing 60 mM NaH_2CO_3, 1 mM $MgCl_2$ (pH 8.2) to a protein concentration of 2 mg/ml. The solution was kept strictly at 0°C and stirred very gently. The reaction was started by the addition of an equal volume of 6 mM or 12 mM 2,4,6-trinitrobenzenesulfonic acid, respectively. Samples of 0.5 ml were taken at times indicated. Lipids were extracted for 30 s with 10 ml chloroform/methanol (2:1, v/v), 2 ml 0.034% $MgCl_2$ solution were added and phases were separated by brief centrifugation. The aqueous upper layer was removed carefully and the chloroform extract was washed twice with 3 ml water/methanol/chloroform (47:48:2, by vol.). After the addition of carrier lipid the extract was taken to dryness, lipids were dissolved in 0.3 ml chloroform/methanol (2:1, v/v) and aliquots were used for analyses.

Analytical procedures

Phospholipids were separated either by one-dimensional (developing solvent: chloroform/methanol/25% ammonia (50:25:6, by vol.)) or by two-dimensional thin-layer chromatography (developing solvents: first dimension chloroform/methanol/25% ammonia (65:35:5, by vol.) or chloroform/methanol/25% ammonia (50:25:6, by vol.); second dimension chloroform/acetone/methanol/glacial acetic acid/water (50:20:10:10:5, by vol.)). Spots on thin-layer plates were visualized by iodine vapour and scraped off for further analysis.

Phospholipids were quantitated by the method of Broekhuyse [18]. Radioactivity of individual lipids after thin-layer chromatography was measured using Ready Solv HP (Beckman)/5% water as a scintillation cocktail.

Sterols were analyzed by gas-chromatography (column 3% OV 101 on Chromosorb 80–100, column temperature 260°C, detector and injector temperature 300°C) after alkaline hydrolysis of the lipid extract with 2% ethanolic KOH for 60 min at 80°C. Stigmasterol was added as an internal standard.

Proteins were quantitated by the method of Lowry et al. [19].

Preparation of phospholipid vesicles

Lipids of yeast membranes were extracted according to the method of Folch et al. [20] using chloroform/methanol (2:1, v/v) as a solvent. A total amount of 3 mg lipid was dispersed by vortexing in 3 ml 10 mM Tris-HCl (pH 7.4). The resultant aqueous lipid suspension was sonicated with a 4 mm soniprobe (25 kHz, 70 W) for 10 min

at 0°C. Titanium particles were removed by centrifugation in a table top centrifuge at approximately 5000 × *g* for 5 min.

Fluorescence measurements

Maximum absorbances of 0.3 at 360 nm were adjusted for all membrane preparations. After the addition of diphenylhexatriene (DPH) (3 μl of a 100 μM solution in tetrahydrofuran) to the sample (3 ml) the labelled mixtures were incubated at 30°C for 30 min; the final concentration of the probe was 0.1 μM. Samples were kept in the dark as long as possible. Fluorescence measurements were carried out using a Shimadzu RF 540 spectrofluorimeter equipped with polarizers in the excitation and emission light path. Constant temperatures in the cuvettes were maintained with a cuvette holder thermostated by an external water bath. Excitation and emission wavelengths were 360 (10 nm slit width) and 430 nm (30 nm slit width), respectively. The measured fluorescence intensities were corrected for background fluorescence and light scattering from the unlabelled sample. The fluorescence anisotropy *r* was determined according to

$$r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2 \cdot G \cdot I_{VH}} \qquad G = \frac{I_{HV}}{I_{HH}}$$

I_{VV} and I_{VH} are the fluorescence intensities determined at vertical and horizontal orientations of the emission polarizer, respectively, when the excitation polarizer is set in the vertical position. I_{HV} and I_{HH} are the fluorescence intensities determined at vertical and horizontal positions of the emission polarizer when the excitation polarizer is set horizontally, *G* is a correction factor for the different response of the monochromators to vertically and horizontally polarized light.

Materials

Zymolyase 20000 was from Kirin Brewery, Tokyo (Japan), TNBS from BDH (Dorset, U.K.) and radiochemicals from Amersham International, U.K.

Results

Lipid composition of mitochondrial membranes

Preparations of the outer mitochondrial membrane were enriched 5–6-fold over whole mitochondria. The cross-contamination of the outer mitochondrial membrane with other cellular membranes was low (Table I) as judged by the measurement of marker enzymes. These results were confirmed by immunotitrations and by the protein pattern of respective fractions (not shown).

TABLE I

PURITY OF MITOCHONDRIAL FRACTIONS

Mitochondria and submitochondrial fractions were isolated as described in Materials and Methods. Microsomes are the 150000*g* pellet from the postmitochondrial supernatant. Data are the mean values from at least three experiments.

	Relative specific activity		
	kynurenine hydroxylase	succinate dehydrogenase	NADPH–cyt-*c* reductase
Mitochondria	1	1	0.07 ± < 0.01
Inner mitoch. membrane	0.68 ± 0.02	1.88 ± 0.06	0.05 ± < 0.01
Outer mitoch. membrane	5.26 ± 0.26	0.15 ± 0.01	0.10 ± < 0.01
Microsomes	0.01 ± < 0.01	0.02 ± < 0.01	1

The phospholipid to protein ratio in the outer mitochondrial membrane is higher (approximately 4-times) than that of the inner membrane (Table II). The only marked difference in the phospholipid composition of the two membranes is the 2–3-fold larger amount of cardiolipin in the inner as compared to the outer membrane. The ergosterol to protein ratio of both mitochondrial

TABLE II

LIPID COMPOSITION OF MITOCHONDRIAL MEMBRANES

	Outer membrane	Inner membrane
Phospholipid : protein (mg/mg)	1.200	0.348
Ergosterol : protein (mg/mg)	0.025	0.024
Ergosterol : phospholipid (mg/mg)	0.021	0.070
% of total phospholipids		
Phosphatidylcholine	44 ± 2	37 ± 1
Phosphatidylethanolamine	34 ± 1	24 ± 1
Phosphatidylinositol	14 ± 1	14 ± 1
Phosphatidylserine	4 ± 1	3 ± 1
Cardiolipin	5 ± 1	13 ± 1
Phosphatidic acid	1 ± 1	2 ± 1

membranes is very similar, but it is higher than it has been described by Bottema and Parks [9]. An approximately 3–4-fold higher ergosterol to phospholipid ratio can be calculated for the inner as compared to the outer membrane.

Transmembrane orientation of phospholipids within the outer mitochondrial membrane

The following methods are commonly used to measure the asymmetric arrangement of phospholipids in bilayer membranes: (1) selective degradation of the outer leaflet of membrane preparations by phospholipases under very mild conditions; (2) exchange of phospholipids of the outer leaflet of a membrane against lipids of external donor vesicles with the aid of phospholipid transfer proteins; (3) chemical modification of phospholipids of the outer leaflet of membranes with non-penetrating chemical reagents; (4) reaction of phospholipid-specific antibodies with phospholipids due to their accessibility on the outer surface of membranes.

Our attempts to apply phospholipase treatment to asymmetry measurements of the outer mitochondrial membrane failed. Incubations of membrane vesicles with phospholipases A_2 or C under various conditions always led to a complete degradation of membrane phospholipids. Recently Manella [21] presented results concerning the disturbance of the structure of the outer membrane of *Neurospora* mitochondria by phospholipases. A similar situation in *Saccharomyces* might explain our inability to employ selective degradation of phospholipids for asymmetry studies.

Phospholipid transfer proteins from bovine heart, bovine liver and from yeast, on the other hand, turned out to be useful tools to determine phospholipid asymmetry of outer mitochondrial membrane vesicles. As can be seen from Fig. 1A 48% of outer membrane phosphatidylcholine were readily exchangeable; more or less identical results were obtained with all three types of phospholipid transfer proteins. With the aid of the bovine heart phosphatidylcholine/phosphatidylinositol specific transfer protein 53% of the outer membrane phosphatidylinositol were transferred to acceptor vesicles. The same result was obtained with the corresponding protein from yeast. Only 24% of the outer membrane phosphatidylethanolamine were readily exchangeable employing a non-specific lipid transfer protein from bovine liver (Fig. 1C) indicating a rather asymmetric distribution of this lipid in the bilayer.

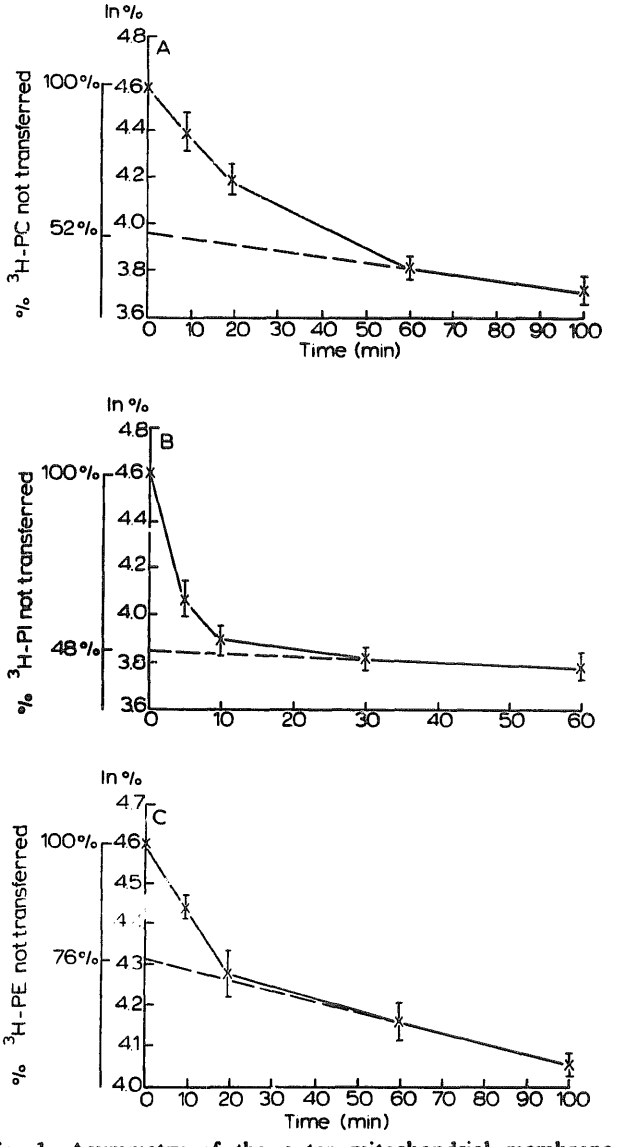

Fig. 1. Asymmetry of the outer mitochondrial membrane. Exchange of phospholipids from the outer leaflet by phospholipid transfer proteins. (A) Phosphatidylcholine transferred by a phosphatidylcholine/phosphatidylinositol specific transfer protein from bovine heart. (B) Phosphatidylinositol transferred by a phosphatidylcholine/phosphatidylinositol specific transfer protein from bovine heart. (C) Phosphatidylethanolamine transferred by the non-specific lipid transfer protein from bovine liver. Assay conditions are outlined in Materials and Methods. Data are results from three experiments. The reproducibility is indicated by error bars.

The exchange of phosphatidylinositol (Fig. 1B) from the outer mitochondrial membrane vesicles

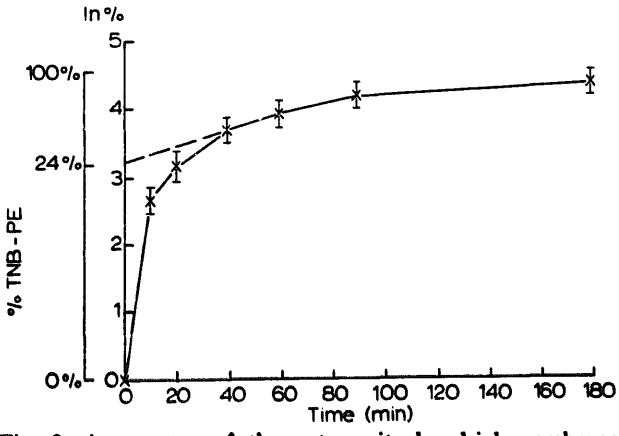

Fig. 2. Asymmetry of the outer mitochondrial membrane: reaction of exposed phosphatidylethanolamine with 2,4,6-trinitrobenzene sulfonic acid (TNBS). Conditions for the incubations of radioactively labelled outer mitochondrial membrane with TNBS are described in Materials and Methods. Values are from two independent experiments. The reproducibility is indicated by error bars.

to acceptor membranes comes to an halt after 10 min, whereas phosphatidylcholine (Fig. 1A) and phosphatidylethanolamine transfer (Fig. 1C) continue during the whole time of the incubation (100 min), although at a slow rate. This might indicate a flip-flop of these lipids across the outer mitochondrial membrane.

2,4,6-Trinitrobenzenesulfonic acid (TNBS) was utilized for the chemical modification of phosphatidylethanolamine of the outer leaflet of the outer mitochondrial membrane. As can be seen from Fig. 2, TNBS reacts with phosphatidylethanolamine very rapidly during the initial phase of the incubation. Depending on the concentration of TNBS (12 or 6 mM, respectively) a break in the time course of the reaction was observed after 10 or 20 min. In the second phase of the incubation phosphatidylethanolamine reacts with TNBS to completeness. We interprete this result as a consequence of a penetration of TNBS into outer mitochondrial membrane vesicles. Results obtained with TNB-labelling of phosphatidylethanolamine confirmed those obtained with the phospholipid transfer protein, indicating that this phospholipid faces predominantly the intermembrane space side of the outer mitochondrial membrane (Table III).

TABLE III

PHOSPHOLIPID ASYMMETRY OF THE OUTER MITOCHONDRIAL MEMBRANE

Results were obtained with radioactively labelled outer mitochondrial membrane. Values were calculated from at least two independent experiments.

	% of individual phospholipid	
	cytoplasmic side	intermembrane space side
Phosphatidylcholine	48±2	52±2
Phosphatidylinositol	53±2	47±2
Phosphatidylethanolamine	24±1 [a]	76±1 [a]

[a] Measured with the aid of a phospholipid transfer protein and by labelling with TNBS.

Fluidity of the outer mitochondrial membrane

The 'fluidity' (lipid aliphatic chain mobility) of the outer mitochondrial membrane was measured by fluorescence spectroscopy using diphenylhexatriene (DPH) as a probe. Anisotropy measurements with this label, which is accommodated in the hydrophobic interior of membranes, allowed a comparison of membrane properties of the outer mitochondrial membrane to that of other yeast membranes, especially the inner mitochondrial membrane and the microsomes.

Measurements carried out at 30 °C, the physiological temperature for growth of yeast cells, showed that the anisotropy of the outer mitochondrial membrane was significantly lower than that of the inner mitochondrial membrane or of microsomes (Table IV). This indicates that phospholipid molecules of the lipid-rich outer mitochondrial membrane are more mobile than those of the protein-rich inner membrane or the microsomes.

TABLE IV

FLUIDITY OF MITOCHONDRIAL MEMBRANES

The fluorescence anisotropy of diphenylhexatriene (DPH) embedded in biological or artificial membranes was measured at 30 °C. Anisotropy was measured with three different membrane preparations.

	Anisotropy
Inner mitochondrial membrane	0.157±0.005
Outer mitochondrial membrane	0.134±0.004
Microsomes	0.152±0.005
Yeast phospholipids plus 2% ergosterol	0.095±0.003
Yeast phospholipids plus 7% ergosterol	0.096±0.003

In order to test if the difference in the ergosterol to phospholipid ratio of the two mitochondrial membranes is the reason for the difference of the fluidity we carried out the following control experiment. Anisotropy of DPH in vesicles consisting of isolated yeast phospholipids plus amounts of ergosterol corresponding to the lipid composition of the outer (2 weight percent ergosterol) and the inner mitochondrial membrane (7 weight percent ergosterol), respectively, was measured. As can be seen from Table IV an increase of ergosterol in this range changes the DPH anisotropy only to a marginal extent.

Discussion

When Bandlow and Bauer [22] described properties of the outer mitochondrial membrane from yeast they showed electron micrographs of isolated vesicles. From these data it became clear that the isolated outer mitochondrial membrane from yeast forms sealed vesicles. This was confirmed by Riezman et al. [5] who proved with biochemical methods, that outer membrane vesicles are sealed and oriented 'right-side-out'. The only report concerning lipids of the yeast outer mitochondrial membrane has been presented by Bednarz-Prashad and Mize [8]. These authors used a preparation technique that was different from the one used in this study and obviously less efficient as judged from marker enzyme measurements and SDS-PAGE patterns. Our preparations of mitochondrial membranes are characterized by the lower percentage of cardiolipin, the higher phospholipid to protein ratio and the lower sterol to phospholipid ratio in the outer as compared to the inner membrane. The latter observation is in contrast to results obtained with mammalian cells, plant cells and also the microorganism *Neurospora crassa* (for a review see Ref. 6). In these types of cells the sterol to phospholipid ratio is up to one order of magnitude higher in the outer as compared to the inner mitochondrial membrane. Yeast cells seem to be an exception with this respect; similar observations had been reported previously by Bottema and Parks [9]. We do not know, however, what the biological relevance of the relatively high sterol content of the yeast inner mitochondrial membrane might be.

The phospholipid transbilayer distribution of an outer mitochondrial membrane has – to the best of our knowledge – not been reported before. The few results concerning the asymmetry of phospholipids in the inner membrane of mammalian mitochondria (summarized in Ref. 6) showed that phosphatidylinositol and cardiolipin (approx. 80% each) seem to be oriented preferentially towards the matrix, whereas phosphatidylcholine and phosphatidylethanolamine are more or less evenly distributed between the two leaflets. Recent works [23], however, showed contradicting results: using adriamycin as a probe similar amounts of cardiolipin were detected on both sides of the inner mitochondrial membrane.

Our attempts to employ different methods to measure the transbilayer distribution were limited by the fact that phospholipases led to a complete degradation of membrane phospholipids, due to phospholipase-induced leakage of the vesicles. Also the chemical modification of aminophospholipids of the outer membrane posed some problems. The outer mitochondrial membrane contains pores [24], and the modifying reagent, TNBS, can penetrate into the lumen of the isolated vesicles. Therefore the reaction of TNBS with aminophospholipids does not come to an halt, but continues after a first phase, during which mainly outer leaflet aminophospholipids are chemically modified. Nevertheless we were able to calculate that approx. 80% of phosphatidylethanolamine is located on the inner aspect of the outer mitochondrial membrane (Fig. 2). This was confirmed by the most suitable method used in our studies of the asymmetry of the outer mitochondrial membrane, the selective removal of phospholipids of the membrane surface by phospholipid transfer proteins (Fig. 1C). This method, which is believed to keep vesicle structures largely intact, also showed that phosphatidylcholine and phosphatidylinositol are more or less evenly distributed over the two halves of the outer membrane bilayer (Figs. 1A and 1B, and Table III).

In spite of the existence of junctions between the outer and the inner mitochondrial membrane the two membranes are different with respect to their protein and lipid composition, and, as a consequence, also with respect to the fluidity of their membrane lipid matrix. Our data (Table IV)

clearly demonstrate that the outer mitochondrial membrane is more fluid than the inner membrane. This could be the result of the higher phospholipid to protein ratio and/or the lower ergosterol to phospholipid ratio of the outer mitochondrial membrane. Control experiments with phospholipid/ergosterol vesicles showed, that the sterol content is of minor influence. From these data we conclude that the large amounts of proteins present in the inner mitochondrial membrane have a stabilizing effect. The inner membrane, which is highly functional with respect to enzyme locations and osmotic behaviour of the mitochondrion, presents itself as a rather rigid membrane. The outer membrane, which communicates with the cytosol and other organelles, is more fluid. This difference in membrane properties between the two membranes might be important for mitochondrial functions insofar as a specific membrane environment is essential for membrane functions.

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