Sterol Analysis of the Inner and Outer Mitochondrial Membranes in Yeast

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ABSTRACT

The membranes of yeast mitochondria were separated and analyzed for lipid content. The sterol-to-phospholipid molar ratio was found to be very similar between the inner and outer membranes (1:30). These observed ratios could be substantially altered by using a crude mitochondrial pellet contaminated with a "floating lipid layer." In this case, the sterol-to-phospholipid molar ratios were 1:8 to 1:26 for the outer and inner mitochondrial membranes, respectively.

INTRODUCTION

Sterols play a prominent role in the fluidity, integrity and structure of eucaryotic membranes. Thus, in organelles such as mitochondria, where membranes control the function of the organelle, it is essential to know how the sterol is distributed. Toward this end, mitochondrial membranes from several systems have been analyzed for their lipid content (1-5). Thus far, no sterol analysis of the inner and outer mitochondrial membranes has been reported for yeast. However, a study with another fungus, *Neurospora crassa,* concluded that sterols were located exclusively in the outer mitochondrial membrane (3). This was inconsistent with our previous experiments in which sterol modifications effected alterations in the Arrhenius plots of the inner mitochondrial membrane enzyme cytochrome oxidase (6). In order to resolve this apparent discrepancy and to ascertain the importance of sterol in yeast mitochondria, we have separated the mitochondrial membranes in *Saccharomyces cerevisiae* and determined the amount of ergosterol present in each membrane with respect to other membrane components.

METHODS AND MATERIALS

Culture Conditions

An isolate of MCC, a wild-type diploid strain of *Saccharomyces cerevisiae,* was grown to late exponential phase (20 hr) at 28 C with aeration in media containing tryptone (1%), yeast extract (0.5%) and ethanol (2% v/v).

Isolation of Mitochondria

Cells were harvested by centrifugation at 5,000 G for 1 min and washed with distilled water (10 ℓ of media typically yielded 60 g wet wt of cells.) The cells were resuspended in 0.5 M β-mercaptoethanol, 0.1 M Tris buffer, pH 9.3, at a ratio of 2 ml buffer/g wet wt of cells and incubated at 28 C with gentle shaking for 5 min. The cells were pelleted (12,000 G, 1 min) and washed twice with a buffer solution at pH 5.8 containing 0.7 M sorbitol, 0.3 M mannitol, 0.1 M citrate, 10 mM K_2HPO_4, and 1 mM EDTA. To form spheroplasts, the washed pellet was resuspended in the same buffer containing 20% Glusulase, an enzyme extract from *Helix pomatia,* at 2.5 ml buffer/g wet wt cells. The suspension was incubated for 1 hr at 28 C with gentle shaking. The spheroplasts were pelleted by centrifugation at 3,000 G for 10 min, washed 3 times in a buffer containing 0.9 M sorbitol, 10 mM Tris, and 0.5 mM EDTA at pH 7.4, and then resuspended in the same buffer at 10 ml/6 g wet wt spheroplasts. The spheroplast suspension was passed through a French pressure cell at 1,000-2,000 psi. Unbroken spheroplasts and cell debris were removed by 3 centrifugations each at 1,100 G for 10 min. To pellet the mitochondria, the supernatant was spun at 12,000 G for 20 min. The mitochondria were resuspended in the above 0.9 M sorbitol buffer and centrifuged at 1,100 G for 10 min to remove the last of the cellular debris. The supernatant was centrifuged once again at 12,000 G for 20 min to recover a crude mitochondrial pellet. This mitochondrial pellet was resuspended in the same buffer, placed on 20-70% linear sucrose gradients and centrifuged for 30 min at 22,700 G in a Sorvall Model SS90 vertical rotor. The gradients were fractionated and each fraction assayed for marker enzymes (see Results). The major band was identified as mitochondrial and was repelleted by centrifugation at 27,000 G for 20 min.

Separation and Isolation of the Inner and Outer Mitochondrial Membranes

In a procedure modified from Neupert and Ludwig (7), the mitochondria were subjected to osmotic swelling and shrinking to enhance the membrane separation, followed by our pro-

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cedure of mechanical shearing through an Eaton pressure cell to strip the outer mitochondrial membrane away from the inner membrane envelope. The whole mitochondria were swollen by resuspending the mitochondrial pellet in 20 mM Tris buffer at pH 8.0 (1 ml buffer/gm wet wt mitochondria) and incubating on ice for 15 min. A solution of 1.8 M sorbitol, 2 mM ATP, 2 mM $MgCl_2$ was then added (0.5 ml sorbitol-ATP-$MgCl_2$ solution/g wet wt mitochondria) and the suspension was left on ice for 1 hr to allow shrinkage of the inner membrane. The mitochondrial suspension was quickly frozen in an Eaton pressure cell and, while still frozen, passed through the small orifice of the cell at 5000 psi. The sheared mitochondria were diluted by adding 2 ml of 20 mM Tris buffer at pH 8.0/g wet wt mitochondria, loaded on 10-20-30-40-50-70% (1:2:2.5:2.5:2.5:1 vol ratios) discontinuous sucrose gradients, and centrifuged at 50,000 G for 1.5 hr. The gradients were fractionated in 1-ml vol from the top and each faction was analyzed for total free ergosterol, phospholipid, protein and enzyme activity.

Quantitation of Sterol, Phospholipid and Protein

The lipids were extracted from each fraction and from whole mitochondria by the Bligh and Dyer method (8) and separated by thin layer chromatography (TLC) using the Skipski et al. procedure (9). Ergosterol was quantitated by gas liquid chromatography (GLC) using cholestane as an internal standard (10). Cholesterol was added during the extraction procedure as a carrier sterol. The quantitation of the recovered cholesterol by GLC also provided a measurement of the efficiency of the sterol extraction.

Phospholipid samples were taken from the lipid extracts described prior to the TLC separation, and were quantitated by the Ames method (11). Protein was determined for each fraction by the Lowry et al. procedure (12). Bovine serum albumin (BSA) was used as the protein standard.

Enzyme Markers

The following enzymes have been localized by Bandlow and Bauer (13) in yeast and were used as marker enzymes to determine the composition of each fraction and the degree of cross-contamination. Rotenone-insensitive NADH-cytochrome c reductase (outer mitochondrial membrane), succinate-cytochrome c reductase (inner mitochondrial membrane) and NADPH-cytochrome c reductase (microsomal) were assayed by the Sottocasa et al. methods (14). Malate dehydrogenase activity (mitochondrial matrix) was measured as described by Vary et al. (15), cytochrome c oxidase activity (inner mitochondrial membrane) as described by Thompson and Parks (16), and kyurenine hydroxylase activity (outer mitochondrial membrane) as described by Schott et al. (17). Chitin synthetase, identified as a plasma membrane marker in yeast (18), was assayed by Cabib's method (19).

Mitochondrial Respiration

Mitochondrial respiration was measured at 28 C in an oxygraph buffer at pH 6.6 containing 15 mM Tris, 15 mM maleate, 0.67 ml phosphoric acid, 1.2 mM EDTA, 6 mM $MgCl_2$ and 0.2% BSA. Ethanol was used as the respiratory substrate.

Materials

Glusulase was purchased from Endo Laboratories. All enzyme assay reagents, L-kyurenine, rotenone, cytochrome c, oxaloacetate, NADPH, BSA, NADH, trypsin, trypsin inhibitor, N-acetylglucosamine and succinate were from Sigma Chemical Co., St. Louis, Mo. UDP-[^{14}C]-N-acetylglucosamine was purchased from New England Nuclear, Boston, MA.

Instrumentation

A Beckman L-65 ultracentrifuge and Sorvall RC-2B centrifuge were used for the centrifugations. The Eaton pressure cell (20) was used in a Carver Model B laboratory press. The enzyme assays were conducted with a PMQ II Zeiss spectrophotometer and the mitochondrial respiration measured on a Gilson oxygraph equipped with a Clark O_2 electrode. GLC separations of sterols were performed with a Varian Series 2700 gas chromatograph equipped with a CDS-111 data processor and a Supelco SP-2250 column.

RESULTS

Assessment of the Purity of Membrane Fractions

The purity and intactness of the mitochondrial preparation is established in Table I. NADPH-cytochrome c reductase and chitin synthetase specific activities (sp act) showed that there was little contamination of the mitochondria with microsomal or plasma membrane protein. The mitochondria also appeared to be intact, exhibiting respiratory competency (RC values of at least 2.5, as calculated from state 3/state 4) and little mitochondrial enzyme release. This fact was important in that mitochondria which were prepared from mechanically broken cells (i.e., shaking with glass beads or disruption in a Bronwill MSK tissue homogenizer) were damaged and thus did not swell or shrink when placed in the anisotonic buffers.

TABLE I

Assessment of the Purity and Intactness of the Mitochondria

Fraction	Malate dehydrogenase (μmol NADH oxidized)	NADPH-Cyt C Reductase (μmol cyt c reduced)	Chitin synthetase (microunits)
	min-mg protein	min-mg protein	mg protein
12,000 G supernatant (microsomes and cytosol)	9.2	24.2	56.1
12,000 G pellet (crude mitochondria)	105.1	0.6	24.5
Major gradient band (purified mitochondria)	168.2	0.0	1.5

Malate dehydrogenase (mitochondrial matrix enzyme) specific activities were calculated from the extinction coefficient of NADH (ϵ = 6.22 x 10^3 $M^{-1}cm^{-1}$) at 340 nm. NADPH-cytochrome c reductase (microsomal enzyme) specific activities were calculated from the extinction coefficient of cytochrome c (ϵ = 19.1 x 10^3 $M^{-1}cm^{-1}$) at 550 nm. One unit of chitin synthetase (plasma membrane enzyme) activity is the incorporation of 1 μmol of acetylglucosamine/min into chitin (19). Enzyme activities were measured as described in Methods and Materials.

Since the swelling and shrinking of the mitochondrial membranes proved necessary for adequate separation, the organelle had to be intact prior to treatment.

Inner and outer mitochondrial membranes from gradient-purified mitochondria could be satisfactorily separated after passage through the Eaton press as demonstrated by the membrane marker enzymes (Table II). The distribution of marker enzyme activities reveal minor cross-contamination of these membranes (Fig. 1). This cross-contamination was on the order of 10%—apparently typical in such separation experiments (7,21,22). Despite repeated attempts, the cross-contamination could not be reduced and other methods of disruption, e.g., sonication (7), homogenization (23) and treatment with ionophors (13), failed to isolate the inner membrane as cleanly.

The gradient fractions containing membrane were identified on the basis of the enzyme markers (Table II). Fraction 1 (0-10% sucrose) had neither inner nor outer mitochondrial membrane enzyme activities. It did, however, contain some protein. Fractions 2-3 (10-20% sucrose) contained the outer mitochondrial membrane. Fractions 4, 5 and 6 (20-30% sucrose) had essentially no assayable enzyme activity but did contain protein. Fractions 7-8 (30-40% sucrose) had both outer and inner mitochondrial membrane enzyme activity and probably represented the formation of mixed vesicles. Compared to the total enzyme yield, however, this fraction was small. The inner mitochondrial membrane was in fraction 9-10 (40-50% sucrose). The density of the inner

TABLE II

Total Activities of Marker Enzymes in Gradient Fractions

Fraction	Cytochrome oxidase (μmol cyt ox)	Succinate-cyt C reductase (μmol cyt red)	NADH-cyt C reductase (μmol cyt red)	Kyurenine hydroxylase (nmol hydrox)
	min	min	min	min
1	1.8	0.5	5.5	0.1
2-3	11.2	1.3	90.6	4.7
4-6	7.3	1.2	6.3	0.2
7-8	19.6	3.3	19.1	0.8
9-10	53.4	7.3	11.8	0.7
11-12	7.3	0.8	1.1	0.2

The inner and outer membranes of gradient-purified mitochondria were separated and identified by marker enzymes. Cytochrome oxidase (inner membrane enzyme), succinate-cytochrome c reductase (inner membrane enzyme), and rotenone-insensitive NADH-cytochrome c reductase (outer membrane enzyme) total activities were calculated on the basis of the cytochromic c extinction coefficient (ϵ = 19.1 x 10^3 $M^{-1}cm^{-1}$) at 550 nm. Kyurenine hydroxylase (outer membrane enzyme) total activities were calculated on the basis that 20 μg hydroxylated kyurenine has an optical density 0.183 units at 492 nm (17). Enzyme assays were conducted as described in Methods and Materials.

mitochondrial membrane was just slightly higher than whole mitochondria run on the same gradients. Fractions 11-12 (50-70% sucrose) had little enzyme activity or protein.

Quantitation of Sterol, Phospholipid and Protein in Membrane Fractions

The analysis of the sterol, phospholipid and protein in each fraction is provided in Table III. The ergosterol-to-phospholipid molar ratio of the gradient-purified whole mitochondria was 1:33. The inner (fractions 9-10) and outer (fractions 2-3) mitochondrial membranes contained similar ergosterol-to-phospholipid molar ratios of 1:30 and 1:29, respectively.

The ratio of ergosterol to protein in whole mitochondria was similar to the inner mitochondrial membrane. The outer membrane, though, had a higher ratio of ergosterol to protein than the inner membrane, as expected since the inner membrane is known to contain more protein than the outer membrane (24).

If the crude mitochondrial pellet (i.e., the mitochondrial pellet prior to gradient purification) was subjected to the separation procedure, a different pattern of sterol ratios was observed as outlined in Table IV. (Note: The crude mitochondria did have the same marker enzyme distribution [Fig. 1] as the gradient-purified mitochondria separations). The ergosterol-to-phospholipid molar ratio of the whole crude mitochondria was significantly higher (1:14) than the gradient-purified mitochondria. The inner and outer membranes also had slightly higher ratios of 1:26 and 1:22. However, the major difference was the appearance of a "floating lipid layer" (fraction 1), which was unobserved in the gradient-purified mitochondria separations. This "floating lipid layer" had extremely high molar ratios of ergosterol to phospholipid (1:2) and ergosterol to protein (0.055 umol/mg), and thus represented a unique fraction.

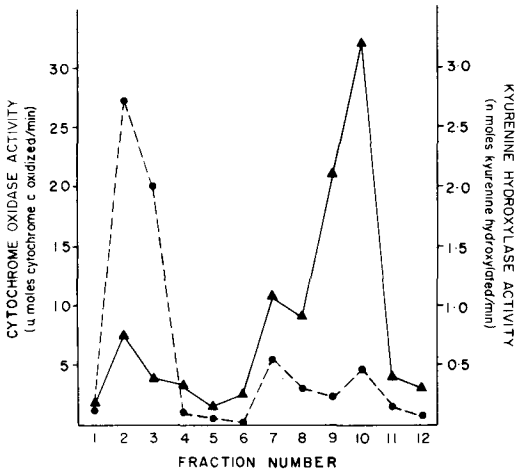

FIG. 1. Total activities of marker enzymes vs gradient fractions. The inner and outer membranes of gradient-purified mitochondria were separated on discontinuous sucrose gradients and identified by marker enzymes. Cytochrome oxidase (—▲—▲—▲—), the inner membrane marker, total activities were calculated from the extinction coefficient of cytochrome c (ϵ = 19.1 x 10^3 M^{-1} cm^{-1}) at 550 nm. Kyurenine hydroxylase (--●--●--●--), the outer membrane marker, total activities were calculated on the basis that 20 μg hydroxylated kyurenine has an optical density of 0.183 units at 492 nm (17). Fraction numbers increase with the density of the gradient, fraction 1 being the top of the gradient. Each fraction represents a 1 ml volume. Preparation of the gradients and enzyme assays are described in Methods and Materials.

DISCUSSION

Our results demonstrate that the ratios of free sterol to the other membrane components

TABLE III

Lipid analysis of the Fractions Derived from Gradient-Purified Mitochondria

Fraction	Sterol/protein (μmol/mg)	Phospholipid/protein (μmol/mg)	Sterol/phospholipid (μmol/μmol)
1	0.0008 ± 0.0001	0.157 ± 0.001	0.0051
2-3 (outer)	0.0142 ± 0.0002	0.416 ± 0.019	0.0341
7-8 (mixed)	0.0094 ± 0.0003	0.232 ± 0.004	0.0405
9-10 (inner)	0.0088 ± 0.0001	0.266 ± 0.008	0.0331
Total	0.0098 ± 0.0005	0.311 ± 0.007	0.0315
Whole mitochondria	0.0094 ± 0.0001	0.313 ± 0.007	0.0300

The mitochondria used for these membrane separations were gradient-purified as outlined in Methods and Materials. The quantitation of the sterol, phospholipid and protein is described in Methods and Materials. All values are expressed as total μmol or mg/gradient graction. The fractions containing membrane were identified on the basis of marker enzyme activities (see text for description). Each entry is the average of 5 experiments and is uncorrected for cross-contamination.

TABLE IV

Lipid Analysis of the Fractions Derived from Crude Mitochondria

Fraction	Sterol/protein (μmol/mg)	Phospholipid/protein (μmol/mg)	Sterol/phospholipid (μmol/μmol)
1 (lipid layer)	0.0546 ± 0.0014	0.108 ± 0.016	0.5056
2-3 (outer)	0.0180 ± 0.0026	0.399 ± 0.020	0.0451
7-8 (mixed)	0.0186 ± 0.0031	0.375 ± 0.019	0.0497
9-10 (inner)	0.0101 ± 0.0012	0.263 ± 0.019	0.0384
Total	0.0233 ± 0.0031	0.275 ± 0.020	0.0847
Whole mitochondria	0.0252 ± 0.0030	0.344 ± 0.031	0.0738

The mitochondria used for these membrane separations were the crude mitochondrial pellets taken prior to gradient purification as described in Methods and Materials. The sterol, phospholipid and protein were quantitated as detailed in Methods and Materials. All values are expressed as total μmol or mg/gradient fraction. The fractions containing membrane were identified on the basis of marker enzyme activities (see text for description). Each entry is the average of 5 experiments and is uncorrected for cross-contamination.

TABLE V

Comparison of Mitochondrial Sterol Ratios from Different Systems

Source	Sterol/phospholipid (μmol/μmol)			Sterol/protein (μmol/mg)			Ref.
	Whole mitos	Outer membrane	Inner membrane	Whole mitos	Outer membrane	Inner membrane	
Rat liver	1:10	1:8	<1:100	0.0160	0.0552	<0.0015	(1)
Rat liver	1:10	1:4	1:29	0.0210	0.0559	0.0087	(25)
Pig heart	1:24	1:15	1:91	0.0120	0.0260	0.0047	(5)
Guinea pig heart	1:39	1:16	1:33	0.0059	0.0778	0.0131	(4)
Neurospora	1:11	1:3	1:32	0.0378	0.4836	0.0126	(3)
Yeast							
Crude	1:14	1:8[a]	1:26	0.0252	0.0355[a]	0.0101	this paper
Purified	1:33	1:29	1:30	0.0094	0.0142	0.0088	this paper

[a]Ratios of the "floating lipid layer" (fraction 1) is included as part of the outer membrane (fractions 2-3).

Mitochondrial sterol ratios from different systems were compiled directly from values reported in the corresponding publication or calculated from the given data. The mitochondrial sterol ratios for yeast were calculated from Tables II and III. Ergosterol: MW = 397; cholesterol: MW = 387; average phospholipid: MW = 700.

do not vary significantly between the inner and outer mitochondrial membranes in yeast. This is incongruous with previous work in rat liver (1,25), guinea pig heart (4), pig heart (5) and *N. crassa* (3) mitochondria, where the sterol-to-phospholipid molar ratios in the outer membranes ranged from 1:3 to 1:16 and the inner membranes ranged from 1:29 to 1:100 (Table V). In these publications, it was concluded that the mitochondrial membranes were very different in sterol composition.

Yeast, then, are distinct from other systems where the outer mitochondrial membrane have been reported to have higher sterol ratios than the inner membrane. Our precise separation and quantitation of sterols, and effective isolation and fractionation of yeast mitochondria support this conclusion. The basis for this difference is the sterol ratios of the outer membrane of yeast mitochondria. Both the ratios of sterol to phospholipid and sterol to protein for the yeast outer membrane are substantially lower than any other published report (Table V). The inner membrane and whole mitochondria of yeast, on the other hand, are comparable to several other systems (Table V). The yeast whole mitochondria, e.g., have sterol ratios similar to pig heart and guinea pig heart mitochondria. The inner membrane of yeast mitochondria contained a significant amount of sterol like many other reports. In some cases (1,5), though, the inner membrane was reported to be free of sterol.

The disparity between previous reports and our present work may be explained on the basis of differences in experimental methods. Reported sterol-to-phospholipid molar ratios can vary widely, even in whole mitochondria from

the same source (e.g., rat liver mitochondria [1,26]). Quantitative techniques for the lipids, particularly the sterols, may be a major cause of these variations. As the amount of sterol esters in the purified yeast mitochondria was extremely small (data not given), sterol esters and precursors were not included in our data as they were in those reports where the Libermann-Burchard assay for sterols or saponification extraction for lipids was employed. Only the "floating lipid layer" from the crude mitochondria showed demonstrable quantities of sterol esters. This contaminating lipid source was eliminated in our preparations.

Another possible explanation as to the disagreement between other reports and our results may lie in the purity of the mitochondria. In our experiments with yeast, gradient-purified mitochondria did not have the "floating lipid layer," which suggests that it is a lipid-rich membrane contaminating crude mitochondrial preparations. In light of the relatively high activity of the plasma membrane marker, chitin synthetase, in the crude mitochondrial pellet, this contaminating lipid may be small plasma membrane vesicles. Since this "floating lipid layer" has a high sterol-to-phospholipid molar ratio, inclusion of this fraction as part of the outer mitochondrial membrane by experimentors would produce a large difference between the sterol ratios of the inner and outer membranes. In particular, this would explain the discrepancy between our results for yeast and those reported for *Neurospora*.

Although some reports indicated that essentially no sterol is present in the inner mitochondrial membrane (1,3,5), we find sterol associated with this membrane. In view of various experiments, including our own, that demonstrated the influence of sterols on inner mitochondrial membrane enzymes (6,25,27), it should be no surprise that free sterols are part of that membrane. Since sterols provide both fluidity and rigidity to membranes, their presence may be necessary in the inner mitochondrial membrane to insure the enzymes a suitable environment in which to function (28).

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