Promitochondria of Anaerobically Grown Yeast. II. Lipid Composition*

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ABSTRACT: The nonrespiring promitochondria of anaerobically grown yeast cells differ from aerobic yeast mitochondria not only in their enzymic equipment, but also in their lipid complement. The most distinctive features of the promitochondrial lipids are their low content of ergosterol and their rather unusual fatty acid composition. Thus, in promitochondria from cells grown in the presence of Tween 80 and ergosterol, oleic acid accounts for approximately 90% of the total unsaturated fatty acids. Promitochondria from cells grown in the absence of a lipid supplement exhibit an extremely low degree of unsaturation and a high content of short-chain ($<C_{14}$) saturated fatty acids. Regardless of the lipid composition of the growth medium, promitochondria contain all of the major phospholipid species typical of aerobic yeast mitochondria, although the relative proportions of phosphatidylinositol and phosphatidylethanolamine are significantly different. The lipid composition of the promitochondria is therefore both similar to, and different from, that of respiring yeast mitochondria.

The results summarized in the preceding paper indicate that anaerobic growth of *Saccharomyces cerevisiae* induces a profound dedifferentiation of the mitochondrial organelles (Criddle and Schatz, 1969). The undifferentiated "promitochondria" accumulating in the anaerobic cells still contain oligomycin-sensitive mitochondrial ATPase (F_1) and mitochondrial DNA but have lost most of the characteristic respiratory pigments as well as a functional electron transfer chain.

The present report describes the lipid composition of the isolated promitochondria. It is well established that lipids rich in unsaturated fatty acids are important structural components of mitochondrial membranes and indispensable for many mitochondrial functions (for review *cf.*, *e.g.*, Fleischer and Fleischer, 1967). Since the synthesis of unsaturated fatty acids in yeast is dependent upon oxygen (Bloomfield and Bloch, 1960), it was reasonable to expect that anaerobic growth would alter the lipid complement of the mitochondrial organelles. Indeed, our data show that the fatty acid composition of promitochondria is quite unique and differs strikingly from that of aerobic, respiring yeast mitochondria.

Materials and Methods

Growth of Yeast Cells and Isolation of (Pro)mitochondria. All experiments described in this paper were carried out with the wild-type *S. cerevisiae* strain D 273-10 B (α Pρ^+, haploid). The growth of the cells and the isolation of (pro)mitochondria were exactly as detailed in the preceding paper (Criddle and Schatz, 1969) except for the following modifications: (1) the anaerobic cultures were routinely poisoned with *both* iodoacetate and cycloheximide prior to harvesting; (2) the aerobic cells were grown in the presence of Tween 80 and ergosterol in order to provide comparable conditions.

Extraction of Lipids from Whole Yeast Cells. The cells were suspended in 0.25 M mannitol–20 mM Tris-SO_4 (pH 7.4)–2 mM EDTA–50 μg/ml of cycloheximide so that each milliliter of the suspension contained 500 mg (wet weight) of cells. The suspension was successively extracted with methanol, chloroform–methanol (2:1), and chloroform (Deierkauf and Booij, 1968). The extracts were combined and purified further according to Folch *et al.* (1957).

Extraction of Lipids from Subcellular Particles. This was carried out with chloroform–methanol (2:1) according to the procedure of Folch *et al.* (1957).

Separation of Lipids. Neutral lipids (including free fatty acids) were separated from phospholipids by thin-layer chromatography on plates coated with 0.5 mm of silica gel H (Merck, Germany). After developing with diethyl ether–petroleum ether (bp 40–60°)–acetic acid (100:5:0.5), the phospholipids remained at the origin. The neutral lipids were recovered by scraping off the silica gel from just above the origin up to the solvent front and extracting three times with chloroform–methanol (2:1). Control experiments with [^{3}H]oleic acid and [^{14}C]-1-heptadecylglycerol indicated that the recovery achieved by this method was essentially quantitative.

The individual phospholipid species were separated from each other by two-dimensional thin-layer chromatography on silica gel H. The solvent system for the first direction was chloroform–methanol–acetic acid–water (50:30:8:4) (Skipski *et al.*, 1964). The second direction was developed with diisobutyl ketone–acetic acid–water (80:50:10) (Marinetti *et al.*, 1957). This procedure resolved the following phospholipids: cardio-

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TABLE I: Lipid Composition of Mitochondria and Promitochondria.

Particle Preparation	Neutral Lipid[a]	Phospholipid (mg/mg of protein[b])	Ergosterol (μg/mg of protein)
Mitochondria (from cells grown in presence of added lipids)	65	0.23	30
Promitochondria (from cells grown in presence of added lipids)	63	0.34	8
Promitochondria (from cells grown without added lipids)	60	0.24	<5

[a] Expressed as micrograms of fatty acids per milligram of protein bound to neutral lipids. [b] Determined as phospholipid phosphorus and calculated on the basis of 40 μg of phosphorus/mg of phospholipids.

lipin plus phosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidylcholine, lysophosphatidylserine plus lysophosphatidylethanolamine, and lysophosphatidylcholine. The various phospholipid spots were identified by comparing their R_F values to those of authentic phospholipid samples. These samples were either synthesized (phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylethanolamine, lysophosphatidylserine, and lysophosphatidylcholine) or obtained commercially (cardiolipin, phosphatidylinositol, and phosphatidylserine). The identity of the phospholipids carrying amino groups was confirmed by staining with ninhydrin. Phospholipids containing choline were also spotted with Dragenforf reagent (*cf.*, *e.g.*, Skidmore and Entenman, 1962) and lipids containing phosphorus with the specific phospholipid spray of Dittmer and Lester (1964). Separation of cardiolipin (possibly contaminated by phosphatidic acid and phosphatidylglycerol) from other phospholipids was also performed by thin-layer chromatography with chloroform–methanol–water (65:25:4) (Wagner *et al.*, 1961). The band corresponding to cardiolipin was scraped off and eluted with chloroform–methanol (2:1). In order to exclude a contamination by phosphatidic acid, an aliquot of the extract was rechromatographed on silica gel H with chloroform–methanol–concentrated NH_3 (70:20:1.5) (de Haas and van Deenen, 1963). The R_F value for cardiolipin was 1.4 relative to that of synthetic phosphatidic acid.

The identity of cardiolipin was further corroborated by chromatography on filter paper impregnated with silicic acid (Marinetti *et al.*, 1957). According to Houtsmuller and van Deenen (1965) this procedure permits an unequivocal distinction between cardiolipin and phosphatidylglycerol.

Fatty Acid Analysis. Phospholipids and neutral lipids were isolated as outlined above and treated with BF_3 and methanol (Morrison and Smith, 1964). The fatty acid methyl esters thus obtained were subjected to gas–liquid partition chromatography in a Packard gas chromatograph equipped with 200 × 0.4 cm glass columns. The stationary phase was 12% ethylene glycol succinate or 10% Apiezon L on Chromosorb W (80–100 mesh). The individual fatty acid methyl esters were identified on the basis of their retention time before and after catalytic hydrogenation (*cf.*, *e.g.*, Burchfield and Storrs, 1962). For quantitative analysis of neutral lipid fatty acids (including free fatty acids), a known amount of heptadecanoic acid was added as an internal standard prior to the thin-layer chromatography.

Miscellaneous Determinations. The quantitative estimation of the various phospholipids after two-dimensional thin-layer chromatography was carried out as follows. The individual spots were visualized by staining with iodine vapor, scraped off, and transferred to Kjeldahl digestion tubes. After mineralization with 0.5 ml of 10 N H_2SO_4, (Parker and Peterson, 1965), inorganic phosphorus was determined according to Bartlett (1959).

Ergosterol was measured on alkaline hydrolysates by the Liebermann–Burchardt reaction (Stadtman, 1957). Protein was estimated by the method of Lowry *et al.* (1951) in the presence of 0.33% sodium deoxycholate. Crystalline bovine serum albumin served as the standard.

Results

Table I summarizes the gross lipid composition of mitochondria and promitochondria. Whereas the various particle types contain almost identical amounts of neutral lipids, they differ from each other by their contents of ergosterol and phospholipid. Thus, promitochondria from anaerobic cells grown in the presence of Tween 80 and ergosterol possess a significantly higher amount of phospholipid and much less ergosterol than aerobic yeast mitochondria. Even less ergosterol is present in the promitochondria from cells grown in the absence of a lipid supplement.

An additional difference between mitochondrial and promitochondrial lipids is revealed by analyzing their fatty acid composition (Tables II and III). In the aerobic mitochondria, the bulk of unsaturated fatty acids is accounted for by approximately equal amounts of oleic and palmitoleic acid. In contrast, promitochondria from cells grown in the presence of a lipid supplement exhibit only very little palmitoleic acid, apparently because its synthesis had been prevented by the lack of oxygen. However, this decrease in palmitoleic acid is almost compensated for by an increased level of oleic acid which now accounts for roughly 90% of the total unsaturated fatty acid. Most, if not all, of the promitochondrial oleic acid undoubtedly stems from the Tween 80 (polyoxyethylene sorbitan monooleate) that had been added to the growth medium.

TABLE II: Fatty Acid Composition of Total Phospholipids.

Particle Preparation	Wt % of Total Fatty Acids							
	C_{10}	C_{12}	C_{14}	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	$C_{20:1}$
Mitochondria (from cells grown in presence of added lipids)	tr	tr	0.6	17.9	43.7	3.6	34.2	tr
Promitochondria (from cells grown in presence of added lipids)	tr	tr	4.5	20.5	6.5	3.9	61.5	3.1
Promitochondria (from cells grown without added lipids)	14.3	8.9	10.4	33.7	12.0	13.7	7.0	tr

TABLE III: Fatty Acid Composition of Neutral Lipids.

Particle Preparation	Wt % of Total Fatty Acids							
	C_{10}	C_{12}	C_{14}	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	$C_{20:1}$
Mitochondria (from cells grown in presence of lipids)	tr	tr	1.2	16.1	43.1	3.6	36.0	tr
Promitochondria (from cells grown in presence of lipids)	tr	tr	4.9	18.0	2.1	10.3	63.0	1.7
Promitochondria (from cells grown without added lipids)	8.5	10.5	14.4	34.0	7.7	17.5	6.4	1.0

TABLE IV: Phospholipid Composition of Mitochondria and Promitochondria.

Particle Preparation	% of Total Phospholipid Phosphorus						
	Cardiolipin	Phosphatidyl-ethanol-amine	Phos-phatidyl-serine	Phos-phatidyl-inositol	Lysophos-phatidyl-ethanol-amine + Lyso-phos-phatidyl-serine	Lyso-phos-phatidyl-choline	Phos-phatidyl-choline
Mitochondria (from cells grown in presence of lipids)	10.9	30.6	4.2	8.1	4.5	3.2	38.5
Promitochondria (from cells grown in presence of lipids)	6.2	19.3	10.0	12.6	4.1	0.3	47.5
Promitochondria (from cells grown without added lipids)	8.9	17.9	3.9	26.0	8.2	0.8	34.3

[a] Not determined.

An even more striking alteration of the mitochondrial fatty acid pattern is induced by anaerobic growth of the yeast cells in the absence of added Tween 80 and ergosterol. The promitochondria isolated from these cells possess only extremely low amounts of unsaturated fatty acids and are unusually rich in short-chain saturated fatty acids of 14 carbon atoms or less. Closely similar results were obtained when the fatty acid composition of the various *intact* yeast cells was analyzed.

The data assembled in Table IV indicate that anaerobic growth also modifies the phospholipid pattern of the mitochondrial membranes. Regardless of the lipid com-

position of the growth medium, promitochondria invariably contain higher proportions of phosphatidylinositol and less phosphatidylethanolamine than aerobic, respiring yeast mitochondria. The other phospholipids exhibit less consistent variations in response to anaerobiosis: the differences between mitochondria and promitochondria are either insignificant (phosphatidylcholine) or observed only in the presence or the absence of a lipid supplement during anaerobic growth (cardiolipin, phosphatidylserine, and lysophosphatidylethanolamine plus lysophosphatidylserine).

The presence of cardiolipin in the isolated promitochondria is of particular interest since this phospholipid is generally considered to be a typical constituent of mitochondrial membranes (*cf.*, *e.g.*, Bartley, 1964). It is of course difficult to exclude that the spot attributed to cardiolipin is in fact some other, as yet unrecognized, phospholipid whose chromatographic behavior closely resembles that of genuine cardiolipin. However, the present data show that this spot is not significantly contaminated by phosphatidic acid or phosphatidylglycerol. Furthermore, lysocardiolipins, prepared from ox heart and promitochondrial cardiolipin with phospholipase A, showed identical chromatographic behavior with the solvent system chloroform–methanol–concentrated ammonia (70:20:1.5) (de Haas and van Deenen, 1963). It seems therefore reasonable to conclude that promitochondria contain most, if not all, of the major phospholipid species which are present in fully developed, respiring yeast mitochondria.

Discussion

The present study shows that the enzymic dedifferentiation of yeast mitochondria during anaerobic growth of the cells (Schatz, 1965; Criddle and Schatz, 1969) is accompanied by profound changes in the lipid composition of the mitochondrial membranes. The most conspicuous features of the promitochondrial lipids are a greatly lowered level of ergosterol and a remarkably simple and unusual fatty acid composition. Especially striking is the very low unsaturation of the promitochondrial lipids from anaerobic cells which had been grown in the absence of added Tween 80 and ergosterol. The residual unsaturated fatty acids are probably derived from the yeast extract of the growth medium. Their concentration within the mitochondrial membranes and the whole cells appears to approach the minimum compatible with survival since staining with methylene blue indicated that up to 40% of the lipid-deficient anaerobic cells were already nonviable. Moreover, attempts to culture anaerobic yeast cells in a synthetic medium devoid of lipid constituents were consistently unsuccessful.

In spite of their unusual lipid complement, the promitochondrial membranes retain the ability to catalyze an oligomycin-sensitive ATPase. This fact agrees with the earlier finding of Kagawa and Racker (1966) that the mitochondrial ATPase complex from beef heart can be reconstituted with a wide variety of rather dissimilar phospholipids. The absence of polyunsaturated fatty acids in promitochondria (as well as in aerobic yeast mitochondria; *cf.* also Bartley, 1964) is quite remarkable since these fatty acids represent up to 50% of the *total* fatty acids of mammalian mitochondria (*cf.*, *e.g.*, Bartley, 1964). It may be profitable to explore the possibility that this difference in lipid composition is causally related to some of the previously observed functional differences between mitochondria from mammals and yeast, *e.g.*, the relatively low sensitivity of yeast mitochondria to oligomycin (Schatz *et al.*, 1967).

The present data are in many ways quite similar to those previously reported for whole anaerobic yeast cells (Kováč *et al.*, 1967; Jollow *et al.*, 1968; Serlupi-Crescenzi and Barcellona, 1966). However, analyses of intact cells do not yield definitive information about the composition of the mitochondrial membranes and may also be ambiguous from a quantitative point of view. Thus, Jollow *et al.* (1968) have recently used the low ergosterol content of lipid-deficient anaerobic yeast as an argument for the absence of mitochondria from these cells. Obviously, this view presupposes that anaerobiosis does not alter the ergosterol content of the mitochondrial membranes. In contrast, our analysis of isolated mitochondria and promitochondria shows that this assumption is incorrect (Table I) and that the conclusion of Jollow *et al.* is no longer tenable.

Whereas the lipid moieties of promitochondria and mitochondria are thus in many respects quite dissimilar, they nevertheless share some important common features. In particular, it appears significant that the phospholipid compositions are qualitatively identical and characteristic for mitochondrial membranes. The present study therefore provides yet another argument for the view that the promitochondria described here are both similar to, and distinct from, the respiring mitochondria of aerobic yeast cells.

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Promitochondria of Anaerobically Grown Yeast. III. Morphology*

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ABSTRACT: Electron micrographs of frozen-etched, anaerobically grown *Saccharomyces cerevisiae* cells reveal the presence of mitochondria-like structures. These nonrespiring "promitochondria" resemble aerobic yeast mitochondria with respect to their characteristic cristae, their double-layered envelope, and their brittle outer membrane which exhibits slits of approximately 100 × 1000 Å. Promitochondria were found regardless of whether the cells had been grown in the presence or the absence of Tween 80 and ergosterol. These observations support the view that anaerobically grown *S. cerevisiae* still contains mitochondrial structures.

The cytology of aerobically grown yeast cells has been extensively investigated and appears fairly well established (Agar and Douglas, 1957; Hagedorn, 1957; Hashimoto *et al.*, 1959; Hirano and Lindegren, 1961; Vitols *et al.*, 1961; Marquardt, 1962; Yotsuyanagi, 1962). In contrast, the structural organization of the anaerobically grown cells has received considerable less attention and is still under dispute. One of the main uncertainties centers around the question of whether the anaerobic cells still contain mitochondrial organelles. In one of the earliest papers on this subject, Linnane *et al.* (1962) reported that anaerobically grown *Torulopsis utilis* cells were devoid of mitochondria and instead contained a pronounced reticular or myelinlike membrane system. These membranes were interpreted as mitochondrial precursors. Absence of mitochondria was subsequently also claimed for anaerobically grown *Saccharomyces cerevisiae* (Wallace and Linnane, 1964; Polakis *et al.*, 1964). In these cells, however, the cytoplasm appeared to be essentially empty and it was therefore proposed that respiratory adaptation of *S. cerevisiae* involved *de novo* formation of the mitochondrial structures (Wallace and Linnane, 1964).

On the other hand, electron micrographs of anaerobic *S. cerevisiae* by Morpurgo *et al.* (1964) suggested that mitochondria were still present, provided the cells had been grown in a medium enriched with Tween 80 and ergosterol. The isolation of mitochondria-like subcellular particles from these anaerobic, lipid-supplemented cells (Schatz, 1965) further supported this view. Wallace *et al.* (1968) have therefore recently modified their original opinion concerning the effect of anaerobic growth on the mitochondrial organelles. They now admit that anaerobic yeast cells are not necessarily devoid of mitochondria but they still maintain that anaerobic growth in the absence of added lipids induced a complete loss of the mitochondrial structures.

The morphological study reported here was prompted

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