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Identification of Mitochondrial Inner Membranes in Anaerobically Grown Baker's Yeast

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The facultative anaerobe *Saccharomyces cerevisiae* (baker's yeast) represents a unique experimental system for studying the mechanisms governing mitochondrial formation. If grown aerobically on a nonfermentable carbon source, this organism contains numerous well-defined mitochondria closely resembling those of mammalian cells (1–3). However, baker's yeast can also meet its

energy requirement exclusively by fermentation and will therefore tolerate extensive modifications of its mitochondrial organelles. For example, growth of the yeast cells in the presence of chloramphenicol (4) or high concentrations of glucose (5, 6) results in the formation of abnormal mitochondria which are deficient in the cytochromes aa_3, b, and c_1 and which have therefore lost most or all of their respiratory capacity. An even more drastic impairment of mitochondrial function is effected by the nonchromosomal "petite" mutation (7). In all these cases, however, the respiration-deficient yeast cells still retain mitochondrial structures and at least some mitochondrial pigments such as cytochrome c.

The most extensive modification of the respiratory apparatus in yeast appears to be induced by growth in the absence of oxygen (1). The anaerobically grown cells exhibit no cyanide-sensitive respiration and have lost all of the cytochromes which are normally associated with the mitochondrial inner membrane (1). However, these cytochromes as well as the respiratory capacity are adaptively regained if the cells are aerated in the presence of an energy source (1). Electron microscopical studies in several laboratories seemed to indicate that the anaerobically grown cells lacked discernible mitochondrial structures (8, 9); Wallace and Linnane proposed, therefore, that yeast mitochondria were completely lost during anaerobic growth and assembled *de novo* during respiratory adaptation (8). In contrast, Morpurgo et al. (10), and Schatz (11) presented suggestive evidence that anaerobic yeast cells grown in the presence of Tween 80 and ergosterol still contained mitochondrial organelles. However, the identification of mitochondria in these yeast cells was not conclusive and the hypothesis of Wallace and Linnane therefore became widely accepted (cf. e.g. references 12, and 13).

The experiments described here were prompted by recent advances in the characterization of the mitochondrial inner membrane (cf. reference 14 for review). These advances have permitted us to conclusively identify mitochondrial inner membranes in anaerobically grown yeast cells and to consolidate and extend our earlier work (11). The present article merely summarizes the main findings of our study; a detailed account will be given elsewhere (15–17).

Experiments with anaerobic yeast cells require the following minimal precautions: (*a*) Maintenance of strict anaerobiosis during cell growth; (*b*) a sufficient number of generation cycles during anaerobiosis; and (*c*) prevention of respiratory adaptation during harvesting of the cells. In our experiments anaerobiosis was ensured by flushing the culture carboys with nitrogen that had been purified by slow passage through a dense suspension of aerobically grown yeast in buffered 2 % ethanol; dilution of the added aerobic cells during anaerobic growth was in the order of 10^4; and respiratory adaptation during harvesting was precluded by cooling the culture to -3 °C, adding an oxygen-free

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solution of cycloheximide (final concentration 20–50 μg/ml), and collecting the poisoned cells in a refrigerated continuous-flow rotor operating in an atmosphere of carbon dioxide.

Low-temperature absorption spectra of the anaerobic cells confirmed the complete absence of cytochromes aa_3, b, c, and c_1 and revealed only small amounts of a pigment with absorption bands at 551.5 and 556 mμ. This pigment is undoubtedly identical with the "cytochrome b_1" described by previous workers (1, 18). The anaerobic cells obtained under our experimental conditions have thus a very simple absorption spectrum and contain at least 20 times less spectroscopically detectable hemoproteins than the corresponding aerobic cells.

On centrifuging a homogenate of the anaerobically grown cells for 90 min

TABLE I

PROPERTIES OF ATPase
ASSOCIATED WITH MITOCHONDRIA-LIKE PARTICLES FROM
ANAEROBICALLY GROWN *S. CEREVISIAE*

Source of particles	ATPase activity (μmoles of ATP cleaved per min per mg protein)			
	No addition	+ F_1 inhibitor	+ F_1 antiserum	Stored 18 hr at 0°C
Wild-type grown with lipid supplement*	1.35	0.12	0.21	1.40
Wild-type grown without lipid supplement	0.71	0.069	0.11	0.78
"Petite" mutant grown with lipid supplement*	0.52	0.078	0.041	0.020

The particles were assayed as described earlier (21) except that they were sonicated for 10 sec prior to being mixed with the various inhibitors.

* The growth medium contained 0.26% Tween 80 and 12 ppm ergosterol.

at 105.000 *g*, 26–30 % of the homogenate protein was recovered in the particulate pellet. Sedimentation of this crude particle fraction in a linear sucrose gradient (20–70 % sucrose; 12 hr at 90.000 *g*) yielded a tight pellet and two main bands at 1.145 and 1.200 g/ml. The particles of the lighter band were studied in more detail since their buoyant density resembled that of aerobic, respiring yeast mitochondria (cf. reference 19). As shown in Table I, the particles of this band exhibited a cold-stable ATPase activity that was sensitive to oligomycin, the F_1-inhibitor of Pullman and Monroy (20) as well as a specific antiserum (21) against purified mitochondrial ATPase from aerobic yeast mitochondria. The ATPase activity was thus clearly a manifestation of coupling factor 1 (F_1) bound to a mitochondrial inner membrane (cf. reference 22).

This conclusion was further supported by investigating the F_1-carrying membranes from anaerobically grown cells of the cytoplasmic "petite" mutant (Table I). These particles contained oligomycin-*in*sensitive and cold-

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labile F_1 and thus exhibited the typical lesion that had earlier been detected (23) in mitochondria of the aerobic "petite" cells.

Other mitochondrial constituents associated with the mitochondria-like particles from the anaerobic yeast cells included flavin, ferrochelatase, as well as succinate- and NADH-dehydrogenase. The dehydrogenases are obviously not linked to a functional respiratory chain as the particles did not catalyze the oxidation of succinate or NADH by molecular oxygen. This fact is not sur-

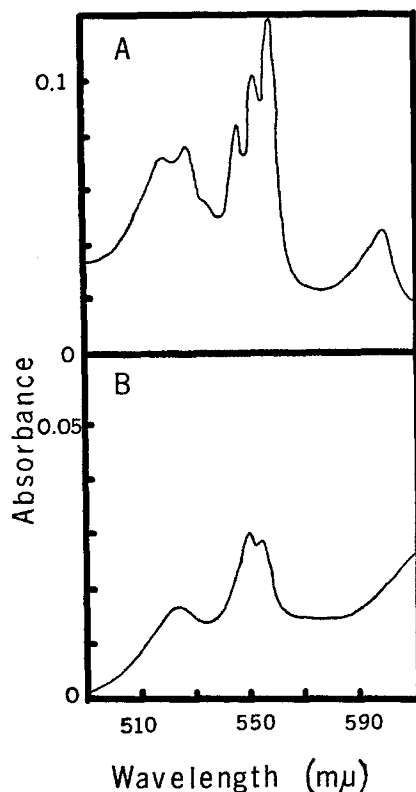

FIGURE 1. Low-temperature absorption spectrum of respiring yeast mitochondria (*A*) and the mitochondria-like particles from anaerobically-grown yeast cells (*B*). The spectra were recorded at the temperature of liquid nitrogen (24) after reduction of the particle-bound pigments with sodium dithionite. The particle concentration in the cuvettes (d = 1 mm) was 2.7 mg/ml (*A*) and 5.4 mg/ml (*B*).

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prising since low temperature absorption spectra of the particles demonstrated the complete absence of the cytochromes aa_3, b, c_1, and c (Fig. 1).

If the F_1-carrying membranes described here are indeed related to the respiring mitochondria of the aerobic yeast cells, they should also contain mitochondrial DNA since this component is one of the most intrinsic earmarks of mitochondrial organelles (12). Our experiments showed indeed that the mitochondria-like particles contained roughly 8 μg DNA/mg particle protein, about two thirds of which was mitochondrial DNA (Fig. 2).

Taken together, these findings would seem to leave little doubt that anaerobically growing yeast cells still form mitochondrial inner membranes. These

membranes are, however, incomplete in that they lack the cytochromes and the organized electron transfer chain associated with the mitochondrial inner membranes of the aerobically grown cells. We therefore propose that the formation of typical yeast mitochondria during respiratory adaptation involves the differentiation of incomplete mitochondria rather than mitochondiral *de novo* formation. Since this view invokes a close analogy between the oxygen-induced synthesis of mitochondria and the light-induced formation of chloroplasts from "proplastids" (cf. reference 26), we have termed the mitochondria-like particles from anaerobically grown yeast "promitochondria." This term

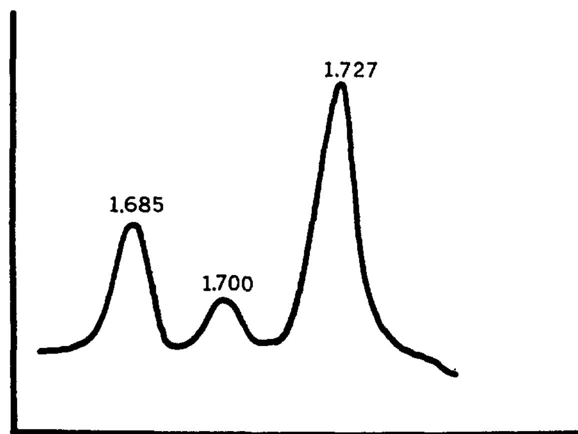

FIGURE 2. Characterization of the DNA associated with the mitochondria-like particles from anaerobically grown *S. cerevisiae*. Densitometric traces of ultraviolet photographs. The DNA sample was centrifuged in a cesium chloride gradient according to Vinograd and Hearst (25). The peak on the left (1.685 g/ml) corresponds to mitochondrial DNA, that in the middle (1.700 g/ml) to contaminating nuclear DNA, and that on the right (1.727 g/ml) to *Pseudomonas aeruginosa* DNA, which served as a reference.

emphasizes the fact that these particles are closely related to the respiring mitochondria of aerobic yeast cells but at the same time lack many of their most distinguishing features.

An anaerobic formation of mitochondrial membranes raises interesting questions regarding their lipid composition. Lipids rich in unsaturated fatty acids are generally considered to be important structural and functional components of mitochondrial membranes (cf. reference 27 for review), yet should not be synthesized in the absence of oxygen (28). The data assembled in Table II show that the fatty acid composition of the promitochondria is indeed quite unique and differs strikingly from that of aerobic, respiring yeast mitochondria. Thus, if the anaerobic cells are grown in a medium enriched with Tween 80 (polyoxyethylene sorbitan monooleate) and ergosterol, oleic acid accounts for approximately 90 % of the total unsaturated fatty acids. The fatty acid pattern

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is even more unusual if the promitochondria from cells grown in the absence of a lipid supplement are analyzed: These membranes exhibit an extremely low degree of unsaturation and a high content of short-chain ($<C_{14}$) saturated fatty acids.

On the other hand, promitochondria contain all of the major phospholipid species found in aerobic yeast mitochondria, although the relative proportions of phosphatidyl inositol and phosphatidyl ethanolamine are significantly altered.

The unusual fatty acid pattern of the promitochondrial membranes could perhaps explain why electron micrographs of chemically fixed anaerobic yeast cells have failed to reveal mitochondrial structures, especially if the cells had

TABLE II

FATTY ACID COMPOSITION OF (PRO) MITOCHONDRIAL PHOSPHOLIPIDS

Particle preparation	Weight per cent 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 aerobically in the presence of added lipids)	Trace	Trace	0.6	17.9	43.7	3.6	34.2	Trace
Promitochondria (from cells grown anaerobically in the presence of lipids)	Trace	Trace	4.5	20.5	6.5	3.9	61.5	3.1
Promitochondria (from cells grown anaerobically without added lipids)	14.3	8.9	10.4	33.7	12.0	13.7	7.0	Trace

been grown in the absence of Tween 80 and ergosterol (8–10). We therefore decided to reinvestigate the cytology of the anaerobic cells by a "physical" fixation method that should be less sensitive to chemical alteration of the mitochondrial membranes. As illustrated in Fig. 3, studies using the freeze-etching procedure of Moor et al. (29) clearly revealed the ultrastructural features of anaerobically grown *S. cerevisiae* and showed numerous promitochondria even in the lipid-deficient anaerobic yeast cells.

If respiratory adaptation does indeed involve differentiation of incomplete promitochondria, then promitochondria isolated from yeast cells after a brief period of respiratory adaptation should contain an exceptionally high concentration of newly synthesized proteins. Results of pulse-labeling experiments with adapting yeast cells have thus far been inconclusive. They do suggest, however, that the majority of the proteins synthesized during the early phases of respiratory adaptation is assembled on cytoplasmic ribosomes and that the

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FIGURE 3. Electron micrograph of a frozen-etched yeast cell grown anaerobically without added lipids. Numerous promitochondria (arrows) are clearly visible. The bar at the lower right denotes 1 μ.

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contribution of a mitochondrial protein synthesizing system is at best very small.

Regardless of these remaining uncertainties it appears justified to conclude that anaerobic growth of yeast does not arrest the synthesis of mitochondrial inner membranes. The postulate of a *de novo* formation of yeast mitochondria during respiratory adaptation (8) is therefore no longer tenable.

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Note Added in Proof. Since this manuscript was submitted we have carried out label transfer experiments which indicate a physical continuity between the promitochondria described here and the respiring mitochondria of oxygen-adapted yeast cells (Schatz, Saltzgaber, and Rouslin, in preparation).

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