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Promitochondria of Anaerobically Grown Yeast, IV. Conversion into Respiring Mitochondria*

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Abstract. Promitochondria of anaerobically grown *Saccharomyces cerevisiae* were selectively labeled *in vivo* by incubating the cells with [^{3}H]leucine and cycloheximide. When the labeled cells were washed free of cycloheximide and adapted to oxygen in the presence of unlabeled leucine, the respiring mitochondria formed during adaptation proved to be radioactive. In contrast, only insignificant label was found in iso-1-cytochrome *c* which is synthesized *de novo* during adaptation. Respiratory adaptation of anaerobically grown yeast thus involves differentiation of promitochondrial organelles.

The facultative anaerobic yeast, *Saccharomyces cerevisiae*, represents an attractive experimental system for studying the biogenesis of mitochondria. If grown aerobically in low concentrations of glucose, the cells possess an active respiratory chain and many well-defined mitochondria. In contrast, the anaerobically grown cells lack a functional respiratory chain but adaptively regain it upon aeration.[1]

Our previous studies have shown that anaerobically grown yeast cells contain mitochondrialike particles.[2] This suggested to us that the formation of functional yeast mitochondria during respiratory adaptation involved the differentiation of incomplete "promitochondria."[2,3] We now describe a label-transfer experiment which demonstrates physical continuity between promitochondria and respiring yeast mitochondria and thus proves a precursor-product relationship between these organelles.

Materials and Methods. Yeast strains: The wild type *Saccharomyces cerevisiae* strains DT XII ($P\rho+$, diploid, ref. 4) and D 273-10B ($\alpha P\rho+$, haploid, ref. 2) as well as the corresponding cytoplasmic "petite" mutants DT XII a and D 273-10B-1 were grown anaerobically to the early stationary phase in the presence of 0.3% glucose, Tween 80, and ergosterol.[2] In a few specified instances, the concentration of glucose was 10% and supplementation of the medium with Tween 80 and ergosterol was omitted. Parallel labeling experiments with the two sets of strains gave essentially identical results.

Labeling procedures: The conditions for the selective labeling of promitochondria *in vivo* with [^{3}H]leucine and the subsequent chase with unlabeled leucine have already been specified in a preliminary communication.[5]

Adaptation of the labeled cells: After labeling, the cells were washed six times at 0°C with 40 mM P_i (pH 7.4)—0.3% glucose—20 mM unlabeled leucine and suspended to 20 mg (wet weight)/ml in ice-cold adaptation buffer (40 mM P_i (pH 7.4)—20 mM unlabeled leucine—1% ethanol and 0.3% glucose). One half of this suspension was shaken for 8 hr at 28°C under nitrogen while the other half was shaken at 28°C in air. The cells were usually broken and fractionated as outlined earlier.[5] In experiments in-

volving electron microscopy, however, homogenates and (pro)mitochondria were prepared by the protoplast method of Kováč *et al.*[4]

Isolation of iso-1-cytochrome *c* from adapted cells: We followed the procedure of Sherman *et al.*[6]

Electron microscopy: *Method A*: Freeze-substitution was conducted as described in reference 7 with the following modifications: (*a*) the substitution medium was ethylene glycol-50% glutaraldehyde (4:1), followed by a graded series of acetone-ethylene glycol mixtures, ending with three changes (1 hr each) in 100% acetone; (*b*) the epoxide resin Durcupan ACM (Fluka AG.) was used for embedding. Sections were stained for 3 hr with 2% aqueous uranyl acetate, followed by 2 min in alkaline lead citrate.[8]

Method B: This method was modified from method 3 of reference 9. Fixation was done at 0°C with 3% acrolein-1% potassium dichromate for 1 hr, followed by 20% acrolein for 1 hr. Samples were dehydrated with a graded series of acetone and embedded and stained as in method A.

For radioautography, anaerobically grown cells were labeled in the presence of [^{3}H]-leucine (59 Ci/mmole; 50 μCi/ml) and of 40 μg/ml cycloheximide essentially as described earlier.[5] After oxygen-adaptation of the washed cells, the mitochondria were isolated[4] and stained for cytochrome oxidase with 2,2'diaminobenzidine and OsO_4.[10] This method proved to be highly specific; only extremely faint membrane images were visible in the micrographs if the incubation was conducted either in the presence of 5 mM KCN, or in the absence of 2,2'-diaminobenzidine, or with unadapted promitochondria. The pellets were then embedded in Epon 812. Autoradiography of the stained specimens was performed by coating pale gold sections with Ilford L-4 nuclear track emulsion and developing with Microdol X.[11] The distribution of silver grains relative to cytochrome oxidase-positive vesicles was analyzed and compared to expected distributions according to the method of Salpeter *et al.*[12] No silver grains (above background) were obtained upon processing stained but unlabeled mitochondrial pellets.

Miscellaneous procedures: Published procedures were employed for measuring Q_{O_2},[13] cell growth,[5] NADH oxidase,[2] F_1-ATPase,[14] protein,[2] and protein-bound radioactivity.[5]

Rationale of Approach. If promitochondria are selectively labeled with [^{3}H]-leucine within anaerobic yeast cells, the fate of this label during subsequent respiratory adaptation in the presence of cold leucine should indicate the mechanism of mitochondrial formation. Thus, mitochondria originating from promitochondria should be labeled since they would receive promitochondrial radioactivity without extensive dilution. Alternately, mitochondria formed *de novo* or from structures other than promitochondria should be unlabeled since the free leucine pool as well as nonpromitochondrial membranes are essentially unlabeled during adaptation.

The label transfer experiment described here was performed with yeast cells that were grown anaerobically in the presence of unsaturated lipids and low concentration of glucose. Mitochondrial development in these cells should be blocked only by lack of oxygen.

Results. Selective labeling of promitochondrial proteins within intact yeast cells: The antibiotic cycloheximide inhibits protein synthesis by the cytoplasmic ribosomal system but has no significant effect on mitochondrial protein synthesis.[1] Therefore, if anaerobically grown yeast cells are exposed to [^{3}H]-leucine in the presence of cycloheximide, only the promitochondrial insoluble proteins[1] should become labeled. Indeed, Table 1 shows that virtually all of the label incorporated into the cells is associated with the promitochondrial fraction.

TABLE 1. *Selective labeling of promitochondria in vivo in the presence of cycloheximide.*

Fraction	Strain DT XII		Strain D 273-10B	
	Protein (cpm/mg)	Total incorporation (%)	Protein (cpm/mg)	Total incorporation (%)
Homogenate	633	(100)	820	(100)
Soluble proteins	76	5.6	87	5.8
Promitochondria	15,850	102	11,800	99

The experiment was performed with anaerobically grown wild type cells. The concentration of promitochondria in the homogenates was determined by ATPase measurements.[2]

In contrast, if the cells are labeled and chased in the absence of cycloheximide, the specific radioactivity of the isolated promitochondria is only about twice that of the soluble proteins. Essentially the same results were obtained with [^{3}H]phenylalanine as radioactive protein precursor.

Since labeling in the presence of cycloheximide is over 100 times slower than in the inhibitor-free controls, it could well reflect labeling by contaminating bacteria or unknown side reactions rather than promitochondrial protein synthesis. However, the following controls render this very unlikely. First, all steps preceding the chase with unlabeled leucine were performed under sterile conditions. Second, isopycnic banding of the labeled crude promitochondria in a sucrose gradient revealed a close correlation between radioactivity and promitochondrial ATPase activity (Fig. 1). Third, *in vivo* labeling of the promitochondria was approximately 90% inhibited by chloramphenicol, a specific inhibitor of mitochondrial protein synthesis.[1] Perhaps the most convincing evidence for the role of promitochondria in this labeling process was the fact that no significant labeling was detected with two nonchromosomal petite mutants lacking a functional mitochondrial genetic system.[15] The very small residual incorporation was insensitive to chloramphenicol and probably reflects incomplete inhibition of the cytoplasmic system by cycloheximide (Table 2). We conclude that our experimental procedure effects a selective labeling of promitochondrial proteins.

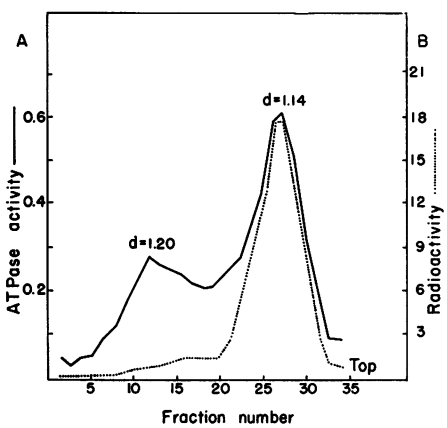


FIG. 1.—Distribution of radioactivity and ATPase activity after centrifuging promitochondria labeled *in vivo*[5] in a sucrose gradient.[2] Strain DT XII was used. *Ordinate A:* ATPase activity (μmole ATP split per 5 min/0.05 ml fraction). *Ordinate B:* protein-bound radioactivity (cpm × 10^3/ml fraction). Most of the ATPase equilibrating at a density of 1.20 g/ml is associated with nonpromitochondrial particles.[2]

Fate of promitochondrial label during respiratory adaptation: When the cells were washed free of cycloheximide and aerated at 28°C in the presence of unlabeled leucine, they rapidly acquired cyanide-sensitive respiration (Table 3). As expected, anaerobically incubated aliquots did not undergo any significant adaptation. These

TABLE 2. *Lack of promitochondrial protein synthesis in a cytoplasmic "petite" mutant.*

Fraction	Control (cpm/mg protein)	Plus chloramphenicol (cpm/mg protein)
Homogenate	174	174
Soluble proteins	38	39
Promitochondria	120	138

Anaerobically grown cells of DT XII a were labeled as outlined under *Materials and Methods* except that one aliquot of the cells was labeled in the presence of cycloheximide *and* 4 mg/ml chloramphenicol. Only the specific radioactivities are given since the impaired binding of mitochondrial ATPase to the mutant promitochondria[2] made it impossible to measure the concentration of these particles in the homogenates.

TABLE 3. *Fate of promitochondrial radioactivity during respiratory adaptation and during incubation under nitrogen.*

	Homogenate	Soluble proteins	(Pro)-mitochondria
Anaerobic cells (Q_{O_2} = 0.4)			
cpm/mg protein	624	72	9340
Total radioactivity (%)	(100)	4.3	101
Cells aerated for 8 hr (Q_{O_2} = 41)			
cpm/mg protein	311	98	4730
Total radioactivity (%)	(100)	5.6	99
Cells incubated for 8 hr under nitrogen (Q_{O_2} = 1.1)			
cpm/mg protein	318	101	4360
Total radioactivity (%)	(100)	6.1	97

The wild type strain D 273-10B was used. The concentration of promitochondria in the unadapted homogenates was determined by ATPase measurements and the concentration of mitochondria in the adapted homogenates by assaying ATPase and NADH oxidase.[2]

aliquots served as a control for protein turnover unrelated to the adaptive process (see ref. 2). Upon fractionation of the adapted cells, over 90% of the cellular radioactivity was concentrated in the mitochondrial fraction. In contrast, iso-1-cytochrome *c* from the adapted cells contained only 270 cpm/mg protein which is only 5.7% of the specific radioactivity of the adapted mitochondria. Since iso-1-cytochrome *c* is synthesized *de novo* during respiratory adaptation,[16] the externally added cold leucine must have effectively diluted the intracellular pool of [^{3}H]leucine and thus prevented reutilization of radioactivity.

This result strongly suggested a physical continuity between promitochondria and respiring mitochondria. However, our evidence at this point was still ambiguous since roughly 50% of the promitochondrial label had been lost during adaptation. *A priori*, this loss might be explained by one of the following mechanisms: (*a*) Differentiation of promitochondria, followed by multiplication of the respiring mitochondria in the unlabeled medium. (*b*) Degradation of (labeled) promitochondrial membranes and simultaneous *de novo* formation of (unlabeled) respiring mitochondria. (*c*) Differentiation of promitochondria and concomitant turnover of (pro)mitochondrial proteins.

The first possibility could be excluded since, under our conditions, respiratory adaptation did not significantly increase the specific activity of F-ATPase of the homogenates (see also ref. 2). The second possibility, too, proved to be unlikely since cells incubated under nitrogen after the initial labeling period lost

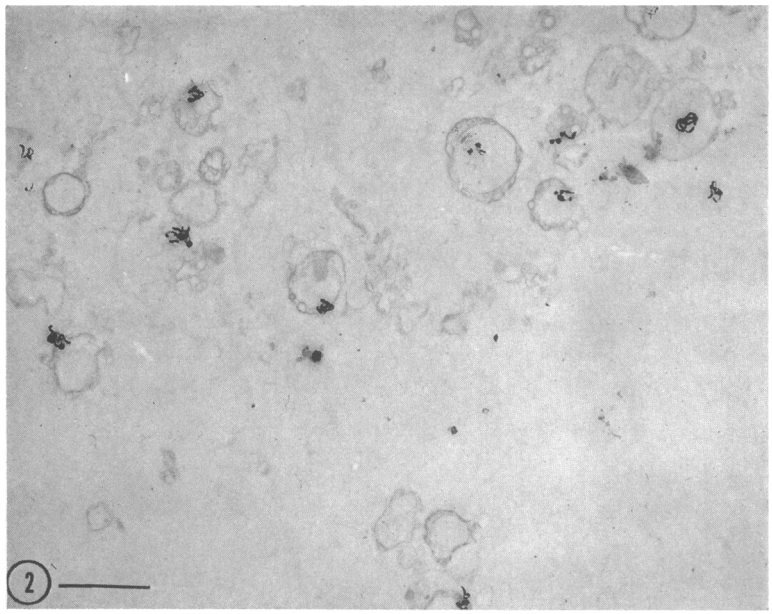


FIG. 2.—Autoradiogram of isolated adapted mitochondria that had been stained for cytochrome oxidase. If radiation spread is taken into consideration,[12] the selective association of silver grains with stained vesicles is readily apparent (see Fig. 3). Bar = 1 μm.

as much radioactivity as cells adapting to oxygen (Table 3). The third possibility thus appeared to be the most plausible one. Nevertheless, it was obvious that rigorous proof for a transfer of promitochondrial label to respiring mitochondria required autoradiography at the level of the electron microscope.

Electron microscope autoradiography: Adapted mitochondrial inner membranes were individually identified histochemically and analyzed for radioactivity by quantitative electron microscope autoradiography. When isolated mito-

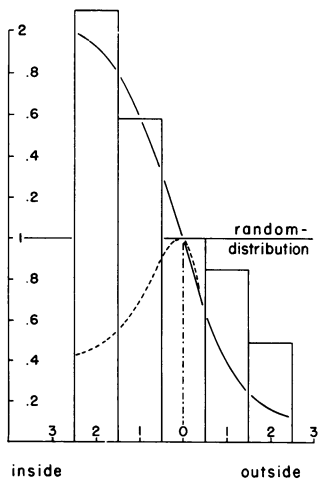
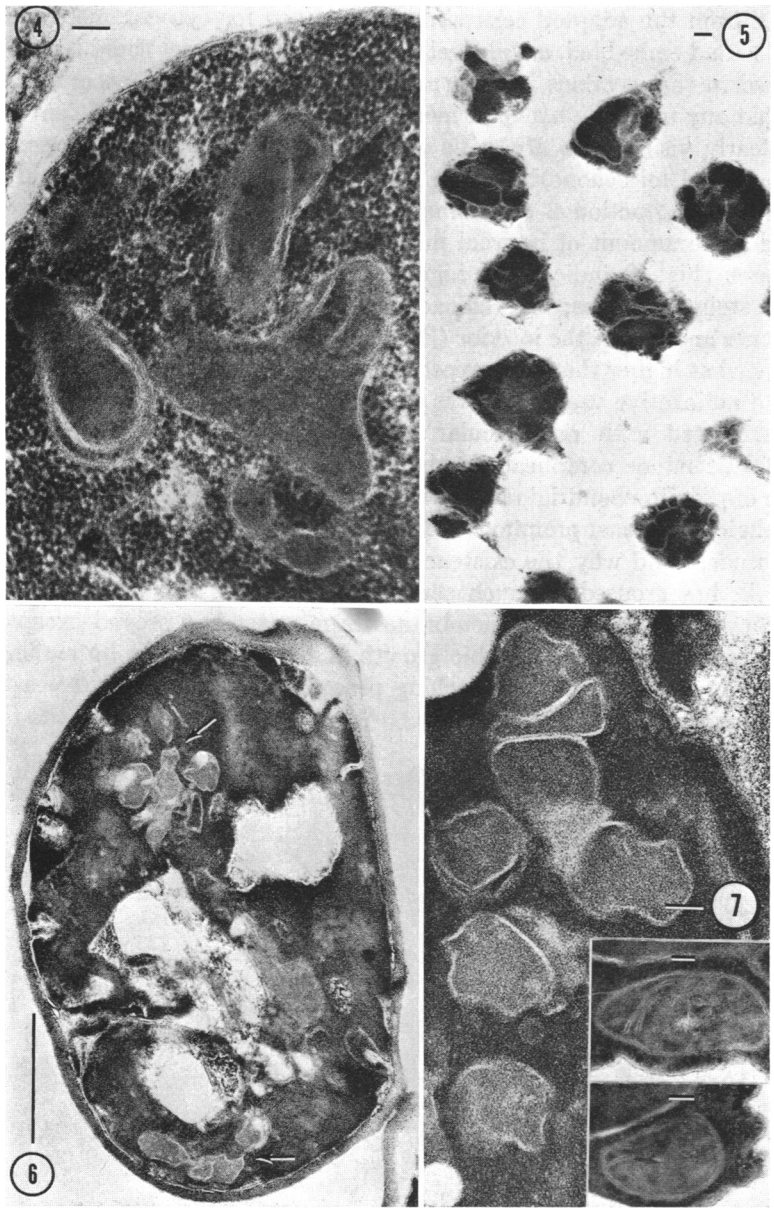


FIG. 3.—Distribution of silver grains around vesicles derived from isolated adapted mitochondria stained for cytochrome oxidase (evaluated by electron microscope autoradiography, see Fig. 2). Experimental density distribution (*bars*) is compared with that calculated for a circular source of similar size labeled only at the periphery (*broken line*) or uniformly throughout (*solid line*). See ref. 12 for details. *Abscissa:* Distance from outer edge of vesicles in units of resolution, HD = 1600 Å. *Ordinate:* Grain density normalized to 1 at outer edge.

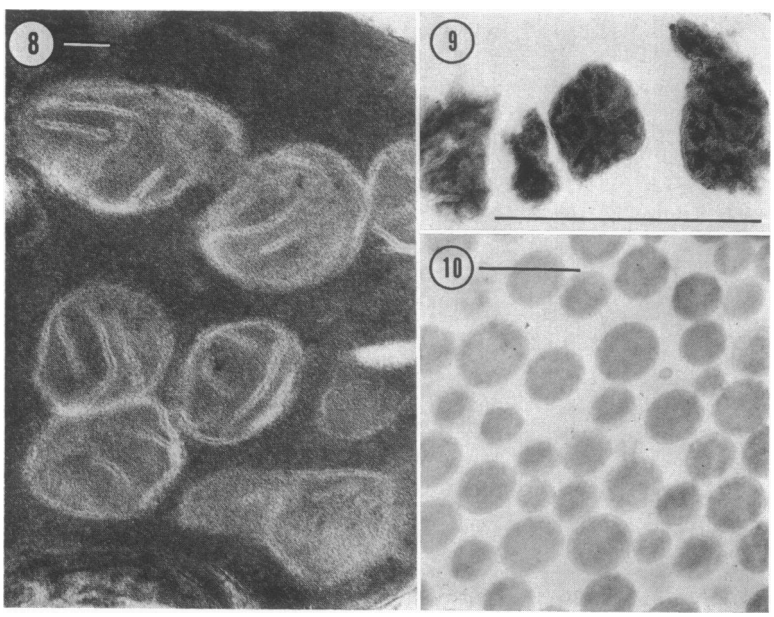


FIGS. 4 and 5.—*S. cerevisiae* (D 273-10B) grown anaerobically in the presence of Tween 80 and ergosterol. Fig. 4: Protoplast (Method A), bar = 0.1 μm. Fig. 5: Isolated promitochondria (Method A), bar = 0.1 μm. Note the frequent circular arrangement of the internal membranes.

FIGS. 6 and 7.—*S. cerevisiae* (D 273-10B) grown anaerobically in the presence of 10% glucose and in the absence of Tween 80 and ergosterol (Method A). The concentration of unsaturated fatty acids in this batch of cells was less than 12% of the total fatty acids.[17] Numerous promitochondria (*arrows*) are seen in the intact cells. After converting the cells to protoplasts, the internal structure of promitochondria can be seen more clearly (insets of Fig. 7). Bars: Fig. 6, 1 μm; Fig. 7, 0.1 μm.

chondria from the adapted cells were first stained for cytochrome oxidase and then fixed and embedded, *adapted* mitochondrial membranes could be selectively visualized in thin sections by electron microscopy. Control experiments indicated that any residual *unadapted* membranes would possess insufficient contrast to be clearly visible (see *Materials and Methods*). While some fragmentation of the adapted mitochondria during the histochemical procedure could not be avoided, a large fraction of the stained material appeared as closed vesicles with a considerable amount of internal membranes. Quantitative analysis of autoradiograms (Fig. 2) indicated a highly significant association of radioactivity with the stained vesicles; the radioactivity was distributed both on the limiting membranes and within the interior (Fig. 3). The grain density outside the rings was somewhat higher than that expected purely on the basis of radiation spread from the radioactive vesicles. This small amount of dispersed label may have been associated with nonvesicular mitochondrial membrane fragments. In spite of this minor complication, the autoradiographic experiments proved a transfer of promitochondrial radioactivity to respiring mitochondria.

Morphology of yeast promitochondria: In retrospect it may perhaps be difficult to understand why the existence of mitochondrial structures in anaerobic yeast cells has aroused so much controversy (see ref. 1). However, electron microscopy of mitochondrial membranes from yeast has proved exceptionally difficult, especially after anaerobic growth of the cells.[3,17] In an earlier study we have shown that the freeze-etching procedure clearly revealed yeast mitochondria under all culture conditions.[3] We have now found methods that are



FIGS. 8–10.—*S. cerevisiae* (D 273-10B) grown anaerobically in the presence of Tween 80 and ergosterol, labeled in the presence of cycloheximide, and adapted to oxygen for 8 hr in the absence of cycloheximide. Fig. 8: Protoplast (Method A), bar = 0.1 μm. Fig. 9: Isolated mitochondria (Method B), bar = 1 μm. Fig. 10: Isolated mitochondria, fixed with OsO_4 and stained with lead citrate. Bar = 1 μm.

equally reliable yet are applicable to ultrathin sections. Either of the two procedures outlined under *Materials and Methods* visualized mitochondrial membranes in negative contrast in almost every cell (Figs. 4–9). Well-defined promitochondrial images could be observed even after mitochondrial development had been maximally repressed by a combination of anaerobiosis, a high concentration of glucose, and the absence of unsaturated lipids. For the sake of comparison, Figure 10 shows that conventional fixation of yeast mitochondria with OsO_4, followed by lead staining, results in very poor membrane contrast. The electron micrographs shown here further support the earlier conclusion[2,3,17] that anaerobically grown yeast cells always retain their mitochondrial structures.

Discussion. The present study shows that the nonrespiring promitochondria of anaerobically grown yeast cells can be transformed into respiring mitochondria. This conclusion rests on a label transfer experiment in which molecules initially present in one type of structure were recovered in another. Although the labeled membrane proteins which served as our markers have not yet been characterized, they are rather insoluble and tightly associated with the promitochondrial inner membrane.[1,19] Their transfer to another membrane type can thus be taken as evidence for physical continuity, especially since a redistribution of label *via* the free leucine pool could be ruled out.

Our data leave little doubt that respiring mitochondria can arise from promitochondria. Although we can not exclude other mechanisms of mitochondrial formation, we consider it rather unlikely that the assembly of functional mitochondria during respiratory adaptation involves the simultaneous operation of different pathways.

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