Distribution of Cytochrome c Peroxidase Activity in Wild-Type and Petite Cells of Bakers’ Yeast Grown Aerobically and Anaerobically

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Studies of mitochondrial biogenesis in yeast have been hampered by a lack of suitable membrane markers in anaerobically grown cells subsequently grown in air. Cytochrome c peroxidase activity and subcellular location was studied to determine whether it would be a useful marker for an analysis of mitochondrial formation. Cytochemical tests revealed enzyme reaction product on all mitochondrial membranes in aerobically grown wild-type cells. Anaerobically grown wild-type and all petite cultures contained cytochrome c peroxidase cytochemical reaction deposits on abundant cytoplasmic membranes and on the few mitochondrial profiles which also were seen in the electron photomicrographs. Biochemical studies corroborated the cytochemistry because mitochondrial fractions were greatly enriched in cytochrome c peroxidase activity for aerobically grown wild-type cultures, but petite and anaerobically grown wild-type cultures showed higher enzyme activities in supernatant fractions than was present in the corresponding particulate fractions after differential centrifugation. Evidence from low-temperature microspectroscopy, spectrophotometric assays of mitochondrial enzyme activities, and electron microscopy showed mitochondrial formation during the time required for preparation and lysis of spheroplasts from anaerobically grown cultures. The data were interpreted as indicating that cytochrome c peroxidase was an oxygen-inducible enzyme, and that there was a developmental relationship between enzyme-reactive membranes of mitochondria and cytoplasm during the period of respiratory adaptation.

The controversy concerning the mode of mitochondrial biogenesis in yeast has continued, despite efforts to establish suitable systems for analysis. Linnane and his co-workers proposed that anaerobically grown yeast cells are amitochondrial (14) and that a new population of organelles appears de novo as a consequence of vesicle transformation (31). In their investigations of respiratory adaptation in yeast, Tustanoff and Bartley (28, 29) demonstrated an amitochondrial state in yeast grown anaerobically on glucose, in agreement with Linnane’s observations. Others have presented ultrastructural and biochemical lines of evidence for the occurrence of fully formed mitochondria (17) or of “promitochondria” (22) in the anaerobically grown yeast cell. Our own preliminary study (21) failed to reveal mitochondrial structures until 50 to 60 min after aeration of cultures which had been grown for the previous 24 hr under anaerobiosis.

Undoubtedly, one of the factors which would influence the results would be the amount of residual oxygen in the anaerobic system. Somlo and Fukuhara (27) showed that trace amounts of molecular oxygen may be sufficient to permit the maintenance of aerobic traits in a culture.

Another problem in yeast studies is inherent in the criteria used for the presence or absence of mitochondria in any developmental stage. Since membrane-bound enzymes such as the cytochromes (31) and succinic dehydrogenase (14) are lacking in anaerobic cells, the usual analyses record the appearance and increase in these parameters as indicators of organelle development. It would be desirable to study a specific mitochondrial membrane marker which underwent little or no change in response to oxygen tension or developmental variation, as an independent means of monitoring the predictable changes in the cytochrome spectra. With such a
marker it also would be possible to compare mitochondrial formation in aerated cultures previously grown under low oxygen tension and to compare biogenesis in wild-type and petite strains which have different organelle ultrastructures (3, 33, 34). The enzyme cytochrome c peroxidase appeared to be such a mitochondrial marker, because it was reported to be active both in aerobic and anaerobic wild-type cultures (10), and our own preliminary cytochemical tests showed enzyme activity in vegetative petite and wild-type strains. The mitochondrial location of cytochrome c peroxidase was reported for aerobically grown Saccharomyces carlsbergensis (32), but comparable studies of S. cerevisiae were obviated by the fragility of the mitochondria. The loss of the enzyme to the supernatant fluid occurs when mitochondria are damaged (32), and this problem may have led others (6, 10) to conclude that the enzyme was nonmitochondrial in S. cerevisiae.

By use of an amine-coupling cytochemical test (5), we successfully localized cytochrome c peroxidase activity product on mitochondrial membranes in bakers' yeast strains. The occurrence of crisp “spots” in wild-type and in petite cells from 24-hr aerobic cultures, and from anaerobically grown cells as well, indicated enzyme activity at the light microscopical level of resolution. Because such spots were shown to be equivalent to mitochondria-containing reaction product in previous studies of cytochrome c oxidase (4), we were encouraged to proceed with an ultrastructural analysis of cytochrome c peroxidase localization. The initial results will be discussed here.

Materials and Methods

Strains. Isogenic diploid strains of S. cerevisiae, which had been studied previously (3, 4), were used in all experiments. The wild-type iso-N and its acriflavine-induced petite derivative DP-28 were maintained at 5 C on nutrient agar slopes containing 1% dextrose. Serial transfers were made at intervals of 4 to 6 weeks.

Growth conditions. The yeast culture was inoculated into 100 ml of semisynthetic (SS) medium (2, 3) in 250-ml Erlenmeyer flasks and grown on a rotary shaker at room temperature for 24 hr with continuous agitation. Such 24-hr cultures were processed for cytochemical tests of enzyme activity in aerobic cultures. For anaerobically grown cells, an inoculum of 10 cells from 24-hr aerobic liquid cultures was added to 150 to 300 ml of fresh SS medium in 500-ml Erlenmeyer flasks. A single 500-ml flask was placed in a Torbal anaerobic jar (Torsion Balance Co., Clifton, N.J.), flushed with H2 gas for 10 min after evaporating the container, and then placed on a rotary shaker at room temperature for 24 hr in the sealed container with an H2 atmosphere. The cells were kept in suspension by gentle rotation on the shaker. Residual traces of oxygen were effectively removed from the jar by reaction with a platinum catalyst secured to the lid inside the container, and the absence of oxygen was monitored by examination of a leuco-base indicator on the jar. After 24 hr of anaerobic growth, the Torbal jar was opened, and the liquid culture was sampled according to the protocol of the particular experiment.

Cytochrome c peroxidase cytochemical test. For each test and control incubation, a 20-ml sample of a 24-hr culture was removed and centrifuged at 23 C to sediment the cells. For 24-hr aerobic cultures, the cells were either washed twice with 0.44 M sucrose, or were used immediately after sedimentation from the liquid growth medium. Because of the urgency of using cells from anaerobic cultures as soon as possible after exposure to air, sucrose washes routinely were omitted in preparing cells for cytochemical tests. Cells were in air from 6 to 8 min between removal from anaerobiosis and immersion in the cytochemical mixture.

Burstone’s (5) method was modified as follows: incubation proceeded at room temperature for 5 to 25 min, depending upon the strain used, in a freshly prepared, freshly filtered mixture containing 1.2 mg of p-aminodiphenylamine and 1.5 mg of p-amino-p’-methoxydiphenylamine dissolved in 0.1 ml of 95% ethyl alcohol; 1.5 ml of 0.2 M tris(hydroxymethyl)-aminomethane (Tris) chloride buffer, pH 7.2 or 7.6; 3.5 ml of glass-distilled water; and 0.025 or 0.1 ml of 3% hydrogen peroxide (H2O2). The product of enzyme activity appeared as purple-black spots in the cytoplasm.

Controls. Incubation mixtures were of two types: lacking H2O2, or complete mixture plus 10 mM potassium cyanide. In most experiments, an additional set of controls consisted of cells permitted to incubate for 2.5 hr in a peroxideless mixture to assess cytochrome c oxidase activity (3).

Electron microscopy. For fine-structure analysis, cells were removed from the reaction mixture at an appropriate time for maximal reaction to have occurred and fixed in unbuffered 5% NaMnO4 after a preliminary wash. The fixing time for aerobic cells was 90 min, whereas 120 to 150 min was required for anaerobically grown cultures. The fixed cells were dehydrated in an acetone series, embedded in Epon 812, and sectioned with a diamond knife on the LKB ultratome. Sections showing a silvery-gray interferences color were picked up on collodion-coated, carbon-stabilized copper grids for scanning in an RCA EMU-3G electron microscope operated at 50 or 100 kv. Photographs usually were taken at an initial plate magnification of 10,000 times. All sections were stained with lead hydroxide (16) to enhance contrast. Samples of cell-free sediments, collected from both strains after centrifugation at 10,000 and 25,000 × g, were prepared for electron microscopy by an osmium method (7) and a glutaraldehyde plus osmium method (23). The latter proved to be more successful, and all photographs were taken of thin sections from Microfuge (23) pellets obtained by this procedure. The sections were stained with lead hydroxide (16) rather than uranyl acetate and lead citrate recommended for
rat liver mitochondria by Schnaitman, Erwin, and Greenawalt (23).

Cell-free extracts. Homogenates were prepared from 16- or 24-hr aerobic cultures grown at room temperature on a rotary shaker with vigorous and continuous agitation. About 10^6 cells from a 24-hr liquid culture were inoculated into 2-liter Erlenmeyer flasks containing 700 to 800 ml of SS medium. After 16 hr for iso-N or 24 hr for野-type, the cells were centrifuged at 2,000 × g for 5 min, washed once with glass-distilled water, and resedimented at room temperature. The washed cells were incubated at 33 C for 30 min in a freshly prepared solution containing 0.14 M mercaptoethylamine-HCl and 0.04 M ethylenediaminetetraacetic acid (EDTA), 2.5 ml per g of cells (7). Then they were sedimented and suspended in 0.22 m sorbitol in 0.02 m KPO_4-EDTA buffer of pH 6.8, 1.7 ml per g of cells; plus Glusulase (Endo Laboratories, Garden City, N.Y.), 1 ml per 10 g of cells; and were then incubated at 33 C for 45 min (L. Grossman, personal communication). The cells were then centrifuged at 2,000 × g for 10 min and suspended in 2 ml per g of cells of a solution containing 0.25 M sorbitol and 1 mm EDTA in 0.05 M KPO_4 buffer of pH 6.8 to be lysed with a Vortex mixer. Lysis usually proceeded for 5 min, after which the brei was centrifuged at 2,000 × g for 10 min. If the resulting supernatant fraction contained no intact cells, as determined with the phase-contrast microscope, the supernatant fluid was centrifuged at 10,000 × g for 20 min. If cells were still present in the supernatant fraction, this fraction was centrifuged again at 2,000 × g for 10 min prior to the high-speed step. The 10,000 × g particles were washed with a solution of 20% sucrose and 1 mm EDTA in 0.05 M KPO_4 buffer of pH 6.8 (7) and sedimented at 10,000 × g for 20 min. At the same time, the supernatant fluid obtained from the first 10,000 × g centrifugation was fractionated further at 25,000 × g for 30 min. The 10,000 and 25,000 × g sediments were solubilized with Zeolite 3A powder (19) and centrifuged to yield an aqueous enzyme extract for assays. The 25,000 × g supernatant fraction was assayed without further treatments. For one experiment, the 25,000 × g supernatant fractions of iso-N and DP-28 aerobic cultures were fractionated further by centrifugation for 3 hr at 100,000 × g in the S40 rotor of the Spinco model L ultracentrifuge.

Cultures which were grown anaerobically, as described above, were fractionated in the same manner as were the aerobic cells. Both strains were grown for 24 hr in the Torbal anaerobic jar before centrifugation to collect the 10,000 × g supernatant at 30,000 × g for enzyme-activity assays.

Except for the initial wash and the sedimentations during the mercaptoethylamine and Glusulase steps, all operations were carried out at 0 to 4 C.

Enzyme-activity assays. The aqueous enzyme extracts and the 25,000 × g supernatant fractions were assayed for cytochrome c peroxidase (EC 1.11.1.7) activity according to Smith's (26) method. The sample cuvette contained 60 mm KPO_4 buffer of pH 6.0 (32), 27 μM ferrocytochrome c, 0.67 mm CH_2OOH, and enzyme extract containing 25 to 50 μg of protein. The reaction was initiated by the addition of enzyme extract and then CH_2OOH to the buffered ferrocytochrome c which had been bubbled previously with carbon monoxide for 2 min. The rate of oxidation of mammalian ferrocytochrome c at 25 C was measured by automatic recording of the decrease in absorbance at 550 μm using 3-ml cuvettes with a 1-cm path-length in a Beckman DB spectrophotometer. Activities were calculated from the initial constant rates and were expressed as first-order rate constants (min^{-1}) per milligram of protein for specific activities and per milligram of total protein in all fractions combined for total activities.

Cytochrome c oxidase (EC 1.9.3.1) activity was assayed according to the spectrophotometric method described previously (4) for these same strains. Specific activities were expressed as first-order rate constants (min^{-1}) per milligram of protein.

The activity of reduced nicotinamide adenine dinucleotide phosphate (NADPH_2)-oxidase was measured by using a modification of the spectrophotometric method of Kovachevich (9). The sample cuvette contained 30 μM NADPH_2, 80 μM ferrocytochrome c, 30 mm KPO_4 buffer of pH 7.3, and enzyme extract containing 25 to 50 μg of protein. NADPH_2 was omitted from the reference cuvette. The reaction was initiated by the addition of the enzyme source, and activity was measured by automatic recording of the change in absorbancy at 340 μm with 3-ml cuvettes with a 1-cm path-length in a Beckman DB spectrophotometer. Activities were expressed as millimicromolecules of substrate consumed (min^{-1}) per milligram of protein (9).

Protein was determined by the colorimetric procedure of Lowry et al. (11) with the Folin-Ciocalteau phenol reagent. Standard curves were established with crystalline bovine serum albumin, fraction V powder.

Reagent chemicals. The sample of CH_2OOH was kindly provided by T. Yonetani. All chemicals were of reagent grade, purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Although the original conditions for the cytochrome c peroxidase (Perox) test differed from those for cytochrome c oxidase (CytOx) only by virtue of the presence of H_2O_2 (5), the cell response was erratic in repeated incubations for Perox, but was consistent for CytOx. To determine the optimal conditions for each strain, the pH of the buffer was varied from 7.2 to 8.1 and the H_2O_2 concentration was modified from 0.01 to 0.1%. The optimal response was judged to be that which occurred in the shortest time, yielding a maximal "spot" count which remained unchanged even on longer incubation time. The optimal conditions proved to be different for the wild-type and petite isogenic strains, and also to be distinct from the CytOx test requirements (Table 1).

Fine-structural localization of Perox reaction product in 24-hr aerobic cultures was different for...
iso-N and DP-28. The wild-type cells contained deposits principally along mitochondrial membranes, including the cristae (Fig. 1). When the incubation was extended deliberately for as long as 2 hr, the deposits invariably were greatly enlarged but still restricted to relatively limited regions of mitochondrial membrane (Fig. 2).

In addition to mitochondrial localization, many cells contained opaque deposits on the few vesicles and membrane strands in the cytoplasm. Frequently, these extramitochondrial deposits were on membranes in close proximity to the plasma membrane (Fig. 3), but never on the plasma membrane itself.

Deposition was quantitatively different for aerobic 24-hr cultures of petite DP-28. In the petite, the reaction product was widely distributed on cytoplasmic membranes as well as on mitochondrial membranes (Fig. 4). Petites usually contain a moderate amount of cytoplasmic membrane, whereas wild-type cells do not (3, 4). Such membranes have been noted in resting cells (30) and in derepressed chloramphenicol-inhibited cells (Clark-Walker and Linnane, in press) of bakers' yeast, and have been found to be characteristic of strongly fermentative yeast strains (15).

In each experiment with 24-hr anaerobic cultures, samples were fixed within 6 to 8 min after the opening of the sealed container. At the same time, other portions of the same cultures were incubated in the Perox reaction mixture and in control systems lacking H₂O₂ or containing 10 mM KCN. Although the untreated cells were in air for a short time, the cytochemically incubated samples were in air for 40 to 60 min prior to fixation in NaMnO₄. Differences in chondriome aspect undoubtedly were due to the length of time that the cells were exposed to air. In the untreated cells, there were one or two low-contrast organelles in a number of cell sections of iso-N (Fig. 5), although most frequently there were no discernible mitochondrial profiles in either strain (Fig. 6 and 7). There were cytoplasmic membranes and vesicles in most of the cell sections from both strains, both for untreated and for cytochemically incubated samples. There was a striking similarity in wild-type and petite cells after Perox incubation, even though petites were fixed 15 to 20 min sooner than the wild type, because of the different test requirements (Table 1). Perox reaction product occurred on mitochondrial and extramitochondrial membranes, but not on the plasma membrane (Fig. 8 and 9). Control cells invariably lacked reaction product deposits (Fig. 10.)

To obtain additional evidence of subcellular sites of Perox activity, we performed spectrophotometric assays of two particle fractions and of the final supernatant fraction after differential centrifugation. In addition to Perox, we assayed for CytoX and NADPH₂-oxidase activities in iso-N, and only for NADPH₂-oxidase in the petite, because CytoX is lacking in respiration-deficient vegetative mutants (4). In one experiment we obtained information on the distribution of succinic dehydrogenase activity (1) in anaerobically grown cells.

Three independent experiments were performed for aerobically grown cultures. Because of the greater ease of obtaining spheroplasts from log-phase or early stationary-phase cultures (7), we used 16-hr iso-N. Previous studies (2) had shown that CytoX activity reached a maximum after 16 hr of growth and that there was no increase in 24-hr cultures. The petite grew for 24 hr before fractionation. The CytoX assays served to identify mitochondrial membranes and NADPH₂-oxidase indicated predominantly microsomal structures (20). The results of the assays are given in Table 2. There was Perox activity in all three fractions from aerobic cultures, but wild-type brei showed nine times higher total activity than the petite. There was higher activity of Perox in DP-28 supernatant fraction relative to DP-28 particles or to iso-N supernatant fraction, in agreement with the fine-structural data concerning cytoplasmic membranes with reaction product deposits in the two strains (Fig. 1 and 4). Unlike Perox, there was no difference in NADPH₂-oxidase activity between iso-N and DP-28. As expected, CytoX activity was confined to iso-N particles.

### Table 1. Bright-field microscopical "spot" counts of 24-hr aerobic cells of iso-N and DP-28 after incubation under optimal conditions for cytochrome c peroxidase and cytochrome c oxidase activities

<table>
<thead>
<tr>
<th>Strain</th>
<th>Respiratory phenotype</th>
<th>Cytochrome c peroxidase</th>
<th>Cytochrome c oxidase</th>
<th>Estimated no. of mitochondria per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% H₂O₂</td>
<td>pH</td>
<td>Time (min)</td>
<td>&quot;Spots&quot; per cell ± se</td>
</tr>
<tr>
<td>iso-N</td>
<td>0.06</td>
<td>7.6</td>
<td>25</td>
<td>43.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>7.2</td>
<td>5</td>
<td>14.5 ± 0.2</td>
</tr>
<tr>
<td>DP-28</td>
<td>Wild type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Petite</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Avers et al. (2, 3, 4).
Fig. 1. Aerobic wild-type iso-N showing nucleus (n), vacuole (v), and cristate mitochondria (m) on which peroxidase cytochemical reaction product can be seen associated with the membranes. X 16,600.

Fig. 2. Enlarged reaction deposits occur on the mitochondria (m) after 2-hr incubation in peroxidase reaction mixture, rather than the optimal 25 min for iso-N. X 16,600.

Fig. 3. Peroxidase cytochemical reaction product on mitochondrial (m) and cytoplasmic membranes (cm), but not on the plasma membrane (pm) in aerobically-grown iso-N. X 37,000.

Fig. 4. Cytochemical localization of peroxidase reaction product on mitochondria (m) and the abundant cytoplasmic membranes (cm) in the petite DP-28 grown aerobically for 24 hr. X 17,100.

Electron micrographs of 10,000 and 25,000 × g particles from both strains showed that occasional protoplasts were present in the slower-speed fraction (Fig. 11), but that most of the structures were isolated mitochondria (Fig. 12 and 14). Numerous membrane-bounded particles were present in the 25,000 × g sediment (Fig. 13). In one experiment, the 25,000 × g supernatant fractions of both strains were centrifuged at 100,000 × g for 3 hr and the particles and supernatant fractions were subsequently assayed for Perox activity. The presence of enzyme activity in
The ultracentrifugate supernatant fraction indicated that some enzyme leakage had occurred, but since activity was present in the 100,000 × g sediment, there was verification of the presence of Perox on cytoplasmic membranes.

The anaerobically grown cultures also contained Perox-active subcellular fractions (Table 2). There was no detectable Perox activity in the 25,000 × g particles of either strain, which contrasted with the subcellular distribution in the aerobically grown cultures. There was considerably more Perox activity in the 25,000 × g supernatant fraction of the anaerobic iso-N culture than in aerobic cultures, in agreement with the ultrastructural cytochemical observations. There was approximately twice the specific activity of NADPH$_2$-oxidase in anaerobic cell fractions, compared with those from aerobic cultures, but no great difference between strains grown under the same environmental conditions (Table 2).

There were clear indications that the chondriome (i.e., the mitochondrial population) of the anaerobically grown cell had undergone developmental changes during the 2.5 to 3 hr required to process the cells for lysis prior to mitochondrial isolation. When removed from the anaerobic jar, the wild-type culture showed no cytochrome a a$_3$ absorption when examined under low-temperature microspectroscopy (25), but there was considerable CytOx activity in the 10,000 × g particles (Table 2). When breis free from whole cells were examined within 30 min after the cultures were exposed to air, no CytOx activity was detectable when measured spectrophotometrically. There was succinic dehydrogenase activity in the

### Table 2. Spectrophotometric assay results from typical experiments showing activities of cytochrome c peroxidase, cytochrome c oxidase, and NADPH$_2$-oxidase in various subcellular fractions obtained by differential centrifugation of aerobic and anaerobic cultures of wild-type iso-N and petite DP-28

<table>
<thead>
<tr>
<th>Strain</th>
<th>Centrifugate fraction</th>
<th>Aerobic*</th>
<th></th>
<th>Anaerobic*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cytochrome c peroxidase</td>
<td></td>
<td>CytOx specific activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total activity</td>
<td>Specific activity</td>
<td></td>
</tr>
<tr>
<td>iso-N</td>
<td>10,000 × g particles</td>
<td>3.54</td>
<td>13.08</td>
<td>16.02</td>
</tr>
<tr>
<td></td>
<td>25,000 × g particles</td>
<td>0.18</td>
<td>2.04</td>
<td>10.92</td>
</tr>
<tr>
<td></td>
<td>25,000 × g supernatant fluid</td>
<td>0.06</td>
<td>0.06</td>
<td>0.00</td>
</tr>
<tr>
<td>DP-28</td>
<td>10,000 × g particles</td>
<td>0.12</td>
<td>4.32</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>25,000 × g particles</td>
<td>0.02</td>
<td>1.98</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>25,000 × g supernatant fluid</td>
<td>0.29</td>
<td>0.30</td>
<td>—</td>
</tr>
</tbody>
</table>

* Specific activities for cytochrome c peroxidase and cytochrome c oxidase are expressed as k (min$^{-1}$)·mg of protein$^{-1}$ and for NADPH$_2$-oxidase as mmoles of substrate consumed·min$^{-1}$·mg·protein$^{-1}$. Total activity of cytochrome c peroxidase is expressed as k (min$^{-1}$)·mg of total protein in all fractions combined$^{-1}$.

b Fractions recovered from the 10,000 × g supernatant fluid upon further centrifugation.

**Fig. 5.** Thin section through iso-N cell fixed 6 min after removal from anaerobiosis. One faint mitochonridion-like image (m) occurs near the nucleus (n). × 17,100.

**Fig. 6.** Iso-N anaerobic cell thin-section showing no mitochondria in the cytoplasm within 6 min of removal from anaerobiosis. × 22,100.

**Fig. 7.** Amitochondrial cell section of the petite DP-28 fixed within 12 min of removal from anaerobiosis. × 17,100.

**Fig. 8.** Distribution of peroxidase reaction product after incubation of anaerobically grown DP-28. Note product on the cytoplasmic membranes (cm) and the mitochondria (m). × 16,600.

**Fig. 9.** Distribution of peroxidase reaction product on cytoplasmic membranes (cm) and mitochondria (m) in anaerobically grown iso-N. × 12,200.

**Fig. 10.** Potassium cyanide control, showing no cytochemical product in iso-N previously grown for 24 hr under aerobic conditions. × 16,100.
10,000 $\times g$ particles from both strains when several hours intervened between exposure to air and eventual spheroplast lysis. In view of these observations, it may be significant that there was considerably reduced activity of cytochrome $c$ peroxidase in anaerobically grown cells of both strains, compared with aerobic cultures (Table 2).

**DISCUSSION**

The electron microscopical observations, supported by activity assays of cell-free fractions, indicated a predominantly mitochondrial location for cytochrome $c$ peroxidase activity in aerobically grown wild-type *S. cerevisiae*. Yonetani and Ohnishi (32) had shown that the enzyme was principally confined to the mitochondria in *S. carlsbergensis*, if care was taken to obtain intact organelles from cell breis. When their mitochondrial fraction was sonic-treated, the peroxidase activity appeared in the supernatant portion of the centrifuged preparation. They (32) further suggested that the earlier reports of an extramitochondrial location by Chantrenne (6) may have been a consequence of the harsh method used for cell breakage. The same interpretation may hold for the results of Lindenmayer and Smith (10), who used the Nossal shaker for cell disruption. We obtained isolated mitochondria from spheroplast lysates produced by gentle agitation on a Vortex mixer after snail enzyme weakening of the cell walls (7), by a method different from that used by Yonetani and Ohnishi (32). With cytochrome $c$ oxidase activity in wild-type cells as an index of mitochondrial membranes, we found no CytOx activity in the 25,000 $\times g$ supernatant fraction, thus substantiating the conclusion that the mitochondria were collected relatively intact for the assays.

The mitochondrial location of Perox is even clearer when the results from anaerobically grown...
cultures are examined. There were occasional organelle profiles visible in cells fixed as quickly as 6 min after exposure to air. There was chondriome development during the time required for preparing spheroplasts prior to lysis. Our observations of anaerobically grown cells a few minutes after exposure to air, showed that cytochrome a a₂ absorption was missing from the low-temperature spectrum, but there was CytOx activity when the mitochondrial particles finally were assayed spectrophotometrically. Interestingly, CytOx activity was confined to the 10,000 × g particles, as was succinic dehydrogenase activity assayed in the same preparations. Since Perox also occurred in the 10,000 × g fraction and not in the 25,000 × g particles, it follows that all three enzymes are mitochondrial in location. In addition, anaerobically grown iso-N cells showed substantially higher Perox activity in the 25,000 × g supernatant fraction, as was predicted from the electron micrographs, which showed abundant cytoplasmic membranes containing reaction product deposits.

One striking feature of anaerobically grown cells of both iso-N and DP-28 was the presence of relatively abundant cytoplasmic membranes, most of which contained Perox reaction product after cytochemical tests, as did the few mitochondria present in the same cell sections. There was independent verification of extramitochondrial membrane localization from enzyme activity assays of subcellular fractions. These data further showed a possible relationship among the Perox-active membranes when comparisons were made of aerobic and anaerobic activity levels. There was 60-fold higher total activity in particles than in supernatant fractions in iso-N aerobic cultures. In contrast, there was higher activity in supernatant than particle fractions in anaerobic iso-N. Since chondriome changes occurred after iso-N anaerobic cultures were exposed to air, we may consider that their cells were in some early stage of mitochondrial formation at the time of organelle isolation, whereas mitochondria from stationary-phase aerobic cultures are considered to be mature (2, 33, 34). Although there are insufficient data, it is tempting to suggest a precursor-product relationship between cytochrome c peroxidase-active cytoplasmic membranes and the mitochondria of the cell. It should be possible to test this hypothesis by analyzing cultures in development during the cell cycle, and others grown under a variety of conditions which influence chondriome development and numerical size (8, 20, 27). Kinetic data would be required to distinguish among the several hypotheses of mitochondrial biogenesis (12, 13, 22, 31). Also, it would be crucial to know if the anaerobically grown cell truly is amitochondrial (14, 22). Our own data are insufficient since amitochondrial cells were obtained in some experiments (21) but not in all. Even though there were occasional faint images which might have been mitochondria, these same 24-hr cultures contained no cytochrome a a₂ as measured spectrophotometrically and spectrophotometrically within a short time after aeration.

If we have correctly assumed the onset of chondriome formation in the interim required for mitochondrial isolation from anaerobically grown cultures, then we may interpret the data further as showing that cytochrome c peroxidase is an oxygen-inducible enzyme in yeast. There was a four- to sevenfold decrease in peroxidase total activity in the “anaerobic” cells of DP-28 and iso-N, respectively, as compared with activities of aerobic cultures. Since CytOx formation apparently was induced during this time period, and probably succinic dehydrogenase activity as well, it may be that the significant differences in peroxidase activity were due to similar metabolic events. Chantrenne (6) and Sels (24) both reported the absence of peroxidase activity in anaerobically grown yeast and its subsequent appearance during aerobicism. Contrary evidence was presented by Lindenmayer and Smith (10), who found no difference in specific activities of cytochrome c peroxidase in aerobically and anaerobically grown wild-type S. cerevisiae.

A persistent problem encountered in studies of mitochondrial and respiratory development in yeast is the lack of correspondence among the several kinds of respiratory measurements and methods (4, 18). The present study has shown that some organelle structure may be present immediately after removal from anaerobiosis, but that cytochrome a a₂ may be absent from these same wild-type cells. Furthermore, the technical difficulties in obtaining parallel data from cells of precisely the same developmental stage may obviate the possibility of accurate comparisons. In addition to these complications, there is the problem of providing stringently anaerobic conditions (27), without which studies from different laboratories cannot be compared easily. Despite these difficulties, the several advantageous properties of unicellular, facultatively anaerobic S. cerevisiae render it a favorable organism for analysis of questions concerning the mitochondrion.

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LITERATURE CITED


