Respiratory Adaptation of Anaerobically Grown *Saccharomyces uvarum*: Changes in Distribution of Enzymes

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Subcellular fractionations using metrizamide density gradients revealed intermediary stages of respiratory adaptation of *Saccharomyces uvarum* grown anaerobically in the presence of the density label 16-bromo-9-hexadecenoic acid. Prior to adaptation, activities of malate dehydrogenase and oligomycin-sensitive ATPase were contained within a membrane population at $\rho = 1.20 \text{ g ml}^{-1}$. After 10 min adaptation cytochrome *c* oxidase activity was associated with these membranes, with ATPase-containing membranes at lower densities ($\rho = 1.05$ to 1.14 g ml^{-1}) and with membranes containing malate dehydrogenase at higher density ($\rho = 1.24 \text{ g ml}^{-1}$). After further adaptation these enzymes were associated firstly with two distinct membrane populations at $\rho = 1.17$ and 1.20 g ml^{-1} and finally with a single population of mitochondria at $\rho = 1.16 \text{ g ml}^{-1}$. The significance of these changes is discussed in terms of mitochondrial differentiation. Peroxisomes were evident even in early stages of respiratory adaptation and were well separated from mitochondria in later stages.

INTRODUCTION

Anaerobically grown yeasts change from a fermentative to a respiratory mode of metabolism in response to aeration of the culture. The adaptation proceeds in the absence of cell multiplication (Cartledge & Lloyd, 1973) and provides a suitable system for studying the development of respiratory apparatus. Membraneous particles containing oligomycin-sensitive ATPase (Mg^{2+} -dependent) of anaerobically grown yeasts are thought to differentiate into fully functional mitochondria during respiratory adaptation and are termed promitochondria or mitochondrial precursors (Plattner & Schatz, 1969; Plattner *et al.*, 1970; Watson *et al.*, 1970).

Cartledge & Lloyd (1973) identified several different populations of particles after sucrose gradient fractionation of adapting cells of *Saccharomyces uvarum*, although the particles were not separated by equilibrium density centrifugation. The authors reported complex density changes in particles containing oligomycin-sensitive ATPase and cytochrome *c* oxidase and proposed that assembly of fully functional mitochondria may involve fusion of membranes which do not pre-exist in anaerobically grown cells. After sucrose gradient fractionation of adapting cells of *Saccharomyces cerevisiae*, Somlo & Krupa (1974) distinguished heavy and light membrane populations by differences in ATPase:respiratory enzyme ratios. Only the heavy population was found in the cells prior to adaptation and evidence of continuity between the two populations in adapting cells was presented.

This paper investigates further changes in enzyme distributions during respiratory adaptation of yeast. By taking advantage of the unsaturated fatty acid requirement for growth under anaerobic conditions (Andreasen & Stier, 1954), we have attempted to incorporate the density label 16-bromo-9-hexadecenoic acid into membranes of *S. uvarum* cells.

METHODS

Maintenance and growth. *Saccharomyces uvarum* NCYC 74S was maintained on potato dextrose agar slopes. The complex medium described by Cartledge & Lloyd (1972b) was used for growth, except that 16-bromo-9-hexadecenoic acid (0.04%; added as a 27%, w/v, solution in ethanol) replaced Tween 80. The pH of the medium was adjusted to 4.5 using 1.0 M-HCl.

Starter cultures were grown to mid-exponential phase on a rotary orbital shaker (Gallenkamp) at low r.p.m., using the medium of Duell *et al.* (1964). Batch cultures (1.5 l) were grown with magnetic stirring in 2-litre conical flasks at 30 °C under an atmosphere of nitrogen. Residual traces of oxygen were removed from high-purity nitrogen (Air Products, New Malden, UK) by passage through 3.5 l of a thick suspension of yeast cells, then through 1 l of Fieser's solution (20%, w/v, KOH; 15%, w/v, sodium dithionite; 2%, w/v, sodium anthraquinone-2-sulphonate) and finally through 1 l of water. The medium was sparged (20 to 25 ml nitrogen min⁻¹) before inoculation (> 12 h) and during growth.

Organisms were counted in an Improved Neubauer haemocytometer (Hawksley, Lancing, Sussex, UK) after suitable dilution and mixing to separate clumps. The inoculum was added to give an initial cell concentration of 2.4×10^3 organisms ml⁻¹. Mean generation time in the exponential phase of growth was 1.7 h and the cell concentration in the stationary phase was 9.5×10^7 organisms ml⁻¹. Cultures were harvested in late exponential phase when the population was between 2×10^7 and 4×10^7 organisms ml⁻¹.

Adaptation of cells. Adaptation at 30 °C was in 1.5 l of medium containing (% w/v): Glucose (0.4), yeast extract (0.2), CaCl₂ (0.033), KH₂PO₄ (0.9), MgSO₄ (0.05) and (NH₄)₂SO₄ (0.6), at pH 5.0. Transfer procedures were carried out under an atmosphere of nitrogen and centrifugation was in sealed containers. The medium had been previously sparged with nitrogen for > 12 h.

After 2 h of glucose derepression, equal volumes of cell suspensions were transferred to six 1-litre conical flasks and aeration was by shaking at 300 r.p.m. on a rotary orbital shaker (Gallenkamp). Respiratory adaptation was stopped by the addition of chloramphenicol (1 mg ml⁻¹) and cycloheximide (0.1 mg ml⁻¹).

Harvesting, preparation and disruption of spheroplasts. All centrifugation procedures were carried out at 4 °C. Cells were harvested by centrifugation for 2 min at 6000 g in a 6 × 250 ml rotor of an MSE 18 centrifuge.

Spheroplasts were prepared (using zymolyase) and disrupted as described previously (Jenkins *et al.*, 1983). Whole cells and intact spheroplasts were removed by centrifugation at 1000 g for 5 min. The supernatant, termed the cell extract, was carefully decanted. In experiments investigating the activities of selected enzymes at stages of respiratory adaptation, cell extracts were prepared from whole cells by ultrasonication (MSE Ultrasonicator) for three 1 min periods with intermittent cooling. Whole cells and intact spheroplasts were removed as described previously.

Fractionation of cell extract by zonal centrifugation. Fractionations through metrizamide gradients were carried out as described previously (Jenkins *et al.*, 1983).

Enzyme assays. Spectrophotometric assays were carried out at 30 °C using a Pye-Unicam SP 1800 spectrophotometer. Malate dehydrogenase (EC 1.1.1.37) was assayed as described by Kitto (1969) except that 0.1 M-potassium phosphate buffer, pH 7.8, was used. Cytochrome *c* oxidase (EC 1.9.3.1) was assayed according to Smith (1955) except that cytochrome *c* was previously reduced with dithionite and passed through a G25 Sephadex column. Catalase (EC 1.11.1.6) was assayed as described by Fukui *et al.* (1975) except that 0.02 M-potassium phosphate buffer, pH 7.4, was used. Assay mixtures for ATPase (EC 3.6.1.3) contained 0.1 M-Tris/HCl, pH 9.5, 4 mM-MgCl₂ and 2.4 mM-ATP (disodium salt); inorganic phosphate was measured according to Fiske & Subbarow (1925). In assays of oligomycin-insensitive ATPase, the inhibitor:protein ratio was between 20 and 30 µg mg⁻¹.

Triton X-100 (final concentration 0.01%) was routinely added to enzyme incubation mixtures for malate dehydrogenase and cytochrome *c* oxidase.

Analytical methods and chemicals. Protein was measured by the Lowry method with bovine serum albumin as standard. Exclusion of metrizamide interference in protein determinations was as described previously (Jenkins *et al.*, 1983).

16-Bromo-9-hexadecenoic acid was synthesized in our laboratory from α-9,10,16-tribromopalmitic acid by the method of Blomquist & Holley (1948). The method of Hunsdiecker (1943) was used to synthesize tribromopalmitic acid from aleuritic acid.

Enzyme substrates were obtained from Sigma. Aleuritic acid and hydrogen bromide/acetic acid were obtained from Fluorochem (Glossop, UK). Metrizamide was obtained from Nyegaard (Oslo, Norway) and all other chemicals were from BDH.

RESULTS

The process of respiratory adaptation was followed by measuring activities of cytochrome *c* oxidase and catalase in cell extracts (Table 1). Cytochrome *c* oxidase was not detectable in extracts of anaerobically grown cells even after 2 h of anaerobic glucose derepression, but showed

Fig. 1. Zonal centrifugation of a cell extract of anaerobically grown glucose-derepressed cells. Cell extract (10 ml) containing 186 mg of protein was loaded on the gradient. Centrifugation was at 20000 r.p.m. for 180 min (6×10^6 g-min at the sample zone; $\int_0^t \omega^2 dt = 4.7 \times 10^{10}$ rad² s⁻¹). (a) Protein (○) and OD₅₂₀ (—); (b) Malate dehydrogenase (○); (c) ATPase (●) and oligomycin-insensitive ATPase (□). Specific activities [nmol min⁻¹ (mg protein)⁻¹] of enzymes in the cell extract were as follows (recoveries in parentheses): malate dehydrogenase, 45 (142%); ATPase, 29 (54%). Protein recovery was 96%.

Table 1. Activities of cytochrome *c* oxidase and catalase in cell extracts during glucose derepression and respiratory adaptation of anaerobically grown *S. uvarum*

	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]*	
	Cytochrome <i>c</i> oxidase	Catalase
Glucose-repressed cells	<0.08	<0.05
2 h Glucose-derepressed cells	<0.08	0.5 ± 0.02
Cells respiratory adapted for 90 min	4.5 ± 0.09	3.0 ± 0.04
180 min	23.5 ± 0.06	4.0 ± 0.08
270 min	35.5 ± 0.12	5.5 ± 0.06

* The values presented are the means ± SD of enzyme specific activities obtained from four experiments.

a marked increase during the period of respiratory adaptation. After aeration for 3 h the specific activity of cytochrome *c* oxidase was similar to that reported by Cartledge & Lloyd (1972*a*) for aerobically grown, glucose-derepressed cells. Although catalase was not detectable in extracts of anaerobically grown glucose-repressed cells, some activity was found after anaerobic glucose derepression. Catalase activity increased steadily throughout the 4.5 h respiratory adaptation period to more than 80% of the activity found in aerobically grown glucose-derepressed cells (Cartledge & Lloyd, 1972*a*).

Fig. 2. Zonal centrifugation of a cell extract of 10 min respiratory adapted cells. Cell extract (10 ml) containing 134 mg of protein was loaded on the gradient. Centrifugation was as in Fig. 1. (a) Protein (○) and OD_{520} (—); (b) catalase (○) and malate dehydrogenase (●); (c) cytochrome *c* oxidase (○), ATPase (●) and oligomycin-insensitive ATPase (□). Specific activities [$\text{nmol min}^{-1}(\text{mg protein})^{-1}$] of enzymes in the cell extract were as follows (recoveries in parentheses): catalase, 5.4 (106%); malate dehydrogenase, 204 (142%); cytochrome *c* oxidase, 7.7 (78%); ATPase, 45 (58%). Protein recovery was 113%.

There was no apparent increase in cell numbers during anaerobic glucose derepression. During respiratory adaptation for 4.5 h the cell count increased from 2.5×10^7 to 3.6×10^7 organisms ml^{-1} .

Fractionation of extracts of anaerobically grown cells after glucose derepression and respiratory adaptation

After anaerobic glucose derepression, the profile of OD_{520} (Pye-Unicam SP 1800) of fractions eluted from the zonal rotor showed peaks in both the sample zone and at $\rho = 1.20 \text{ g ml}^{-1}$ (Fig. 1a); the former zone contained approximately 70% of protein and the latter contained most of the remaining protein (Fig. 1a). The sedimentable zone also contained virtually all of the recovered activities of malate dehydrogenase (Fig. 1b) and oligomycin-sensitive ATPase (Fig. 1c).

After respiratory adaptation for 10 min, the profile of OD_{520} was much more complex, with distinct peaks at 1.04, 1.09, 1.11, 1.20 and 1.24 g ml^{-1} (Fig. 2a). The region at $\rho = 1.04 \text{ g ml}^{-1}$ corresponded with the sample zone and contained approximately 60% of both protein (Fig. 2a) and catalase activity (Fig. 2b). Remaining protein was distributed throughout the gradient with no distinct maximum, and sedimentable catalase activity was located in the zone at $\rho = 1.20 \text{ g ml}^{-1}$. The most active fractions of malate dehydrogenase (Fig. 2b), cytochrome *c* oxidase (Fig. 2c) and oligomycin-sensitive ATPase (Fig. 2c) were again found at $\rho = 1.20 \text{ g ml}^{-1}$. Further peaks of malate dehydrogenase and cytochrome *c* oxidase, however, were located at $\rho = 1.24 \text{ g ml}^{-1}$, although little oligomycin-sensitive ATPase was recovered from this region.

Fig. 3. Zonal centrifugation of a cell extract of 30 min respiratory adapted cells. Cell extract (10 ml) containing 76 mg of protein was loaded on the gradient. Centrifugation was as in Fig. 1. (a) Protein (○) and OD_{520} (—); (b) catalase (○) and malate dehydrogenase (●); (c) cytochrome *c* oxidase (○), ATPase (●) and oligomycin-insensitive ATPase (□). Specific activities [$\text{nmol min}^{-1}(\text{mg protein})^{-1}$] of enzymes in the cell extract were as follows (recoveries in parentheses): catalase, 4.8 (96%); malate dehydrogenase, 106 (108%); cytochrome *c* oxidase, 8.9 (73%); ATPase, 47 (45%). Protein recovery was 119%.

Proportions of cytochrome *c* oxidase (20%) and oligomycin-sensitive ATPase (45%) activities were also recovered over densities of 1.05 to 1.14 g ml^{-1} .

After respiratory adaptation for 30 min, the profile of OD_{520} (Fig. 3a) again indicated at least five regions on the gradient, but at densities of 1.04, 1.06, 1.17, 1.19 and 1.25 g ml^{-1} . Non-sedimentable portions of protein (48%; Fig. 3a) and catalase activity (55%; Fig. 3b) were recovered from the zone at $\rho = 1.06 \text{ g ml}^{-1}$. Sedimentable protein showed a complex distribution, particularly over densities of 1.15 to 1.25 g ml^{-1} . Sedimentable activity of catalase was also recovered over these densities with peak activities at $\rho = 1.21 \text{ g ml}^{-1}$. Activities of malate dehydrogenase (Fig. 3b) and cytochrome *c* oxidase (Fig. 3c) were broadly distributed, mainly over densities of $\rho = 1.15$ to 1.25 g ml^{-1} , and showed corresponding peaks of activity at $\rho = 1.19 \text{ g ml}^{-1}$. ATPase activity was also broadly distributed ($\rho = 1.10$ to 1.25 g ml^{-1}) but peak activity of this enzyme was located at the lower density of $\rho = 1.17 \text{ g ml}^{-1}$.

After respiratory adaptation for 180 min, the profile of OD_{520} showed only two peaks: one corresponding with the sample zone and the other at $\rho = 1.16 \text{ g ml}^{-1}$ (Fig. 4a). The sample zone contained 55% of protein (Fig. 4a), 58% of catalase activity (Fig. 4b) and 46% of malate dehydrogenase activity (Fig. 4b). The region at $\rho = 1.16 \text{ g ml}^{-1}$ contained most of the remaining protein, sedimentable activity of malate dehydrogenase and all the recovered activities of cytochrome *c* oxidase and oligomycin-sensitive ATPase (Fig. 4c). Sedimentable catalase activity was recovered mainly over densities of 1.18 to 1.21 g ml^{-1} (Fig. 4b).

Fig. 4. Zonal centrifugation of a cell extract of 180 min respiratory adapted cells. Cell extract (10 ml) containing 239 mg of protein was loaded on the gradient. Centrifugation was as in Fig. 1. (a) Protein (○) and OD₅₂₀ (—); (b) catalase (○) and malate dehydrogenase (●); (c) cytochrome *c* oxidase (○), ATPase (●) and oligomycin-insensitive ATPase (□). Specific activities [nmol min⁻¹ (mg protein)⁻¹] of enzymes in the cell extract were as follows (recoveries in parentheses): catalase, 8 (87%); malate dehydrogenase, 215 (92%); cytochrome *c* oxidase, 29 (87%); ATPase, 21 (57%). Protein recovery was 106%.

DISCUSSION

The growth kinetics of the culture indicate that 16-bromo-9-hexadecenoic acid serves readily as a source of unsaturated fatty acid for anaerobically grown *S. uvarum*. The density-labelled promitochondria of the glucose-repressed cells have a higher equilibrium density in sucrose ($\rho = 1.25 \text{ g ml}^{-1}$; R. O. Jenkins, unpublished results) than those of yeast cells supplemented during growth with Tween 80 ($\rho = 1.23 \text{ g ml}^{-1}$; Cartledge & Lloyd, 1972*b*; Somlo & Krupa, 1974). After anaerobic glucose derepression, respiratory adaptation proceeds rapidly and in the absence of significant net growth of culture.

Separation of organelles was best achieved by fractionation of cell extracts using metrizamide gradients as opposed to the sucrose gradients previously used (Cartledge & Lloyd, 1973; R. O. Jenkins, unpublished results). The equilibrium density of promitochondria in metrizamide was 1.20 g ml^{-1} . After respiratory adaptation for only 10 min, a large proportion of cytochrome *c* oxidase was associated with these organelles. Remaining cytochrome *c* oxidase was associated with particles containing ATPase at low density ($\rho = 1.05$ to 1.14 g ml^{-1}) and particles containing malate dehydrogenase at high density ($\rho = 1.24 \text{ g ml}^{-1}$); these populations were eventually lost or modified during further adaptation. After 30 min the equilibrium density of membranes containing ATPase had shifted from $\rho = 1.20 \text{ g ml}^{-1}$ to $\rho = 1.17 \text{ g ml}^{-1}$, though cytochrome *c* oxidase and malate dehydrogenase remained largely at the higher density. The ratios of oligomycin-sensitive ATPase to cytochrome *c* oxidase at these densities (2.8 and 6.7 respectively)

indicate heterogeneity of membranous populations. After 180 min, ATPase and cytochrome *c* oxidase were contained within mitochondria with an equilibrium density of 1.16 g ml^{-1} .

The conditions employed in these fractionations did not lead to extensive solubilization (<5%) of mitochondrial-membrane marker enzymes. The possibility that newly assembled organelles in the early stages of adaptation are more fragile cannot be discounted, but the finding that malate dehydrogenase (a mitochondrial matrix enzyme) is virtually all sedimentable and forms discrete zones suggests that particles are not produced by organelle comminution. In addition, damage to organelles would not account for the differential ratios of membrane bound enzymes obtained after fractionation of cells adapted for 30 min.

The density shifts in ATPase carrying membrane populations reveal intermediate stages of membrane assembly and point to possible sequential stages of *in vivo* mitochondrial differentiation. The concept of the promitochondrion as a mitochondrial precursor supposes that this organelle acts as a framework into which newly synthesized lipid and enzyme molecules are fitted (Plattner *et al.*, 1970). Whilst our results do not discount this hypothesis, they support the proposal of Cartledge & Lloyd (1973) that mitochondriogenesis may involve fusion of quite large membrane units which are synthesized *de novo* in early stages of respiratory adaptation. After 30 min adaptation, the membrane populations at $\rho = 1.17$ and 1.20 g ml^{-1} may correspond to two distinct mitochondrial populations. Several alternative mechanisms responsible for observed density changes may be proposed. The shift to lower density of membranes occurring in the 3 h adaptation period suggests that the higher density population (even after 30 min) may be a precursor of the lighter one. Alternatively, the shift to lower density may come about through fusion of particles sedimenting to $\rho = 1.10 \text{ g ml}^{-1}$ with other higher density particles. This interpretation is compatible with the phase transition temperature changes observed by Ainsworth *et al.* (1972), which show that enzymes synthesized during adaptation of *S. cerevisiae* become associated with both anaerobically formed membrane and new aerobically formed membranes.

The reasonably constant sedimentable proportions of catalase obtained for the fractionations suggests that peroxisomes are present even in early stages of respiratory adaptation. These organelles show little change of diversity during adaptation and in later stages are well separated from mitochondria.

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