Subcellular Fractionation of *Candida boidinii* after Growth on Glucose or Methanol

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Extracts of glucose-grown and methanol-grown *Candida boidinii* were fractionated on sucrose gradients. Mitochondria and microbodies from methanol-grown cells were more fragile than those from glucose-grown cells. Formaldehyde and formate dehydrogenases and antimycin A-insensitive NADPH:cytochrome *c* oxidoreductase were non-sedimentable enzymes. The mitochondrial zone defined by cytochrome *c* oxidase ($\rho = 1.19$ to 1.20 g ml^{-1}) contained sedimentable activities of malate dehydrogenase and citrate synthase. The vacuolar enzyme acid phosphatase was also recovered from this region. The microbody enzyme catalase sedimented to a lower density ($\rho = 1.17 \text{ g ml}^{-1}$) than mitochondria after growth on glucose but, together with methanol oxidase activity, to higher densities ($\rho = 1.23$ and 1.27 g ml^{-1}) after growth on methanol. Activity of the methanol assimilation enzyme dihydroxyacetone synthase was partially sedimentable and was recovered in the fraction containing mitochondria and membraneous vesicles. Inosine diphosphatase was probably multilocational in the cells.

INTRODUCTION

Growth of various yeasts on methanol is accompanied by increased development of microbodies (van Dijken *et al.*, 1975*a*; Sahm *et al.*, 1975; Tanaka *et al.*, 1976). These organelles are involved in the initial oxidative metabolism of methanol and are also termed peroxisomes (van Dijken *et al.*, 1975*a, b*; Fukui *et al.*, 1975; Roggenkamp *et al.*, 1975; Veenhuis *et al.*, 1976). Microbodies of glucose-grown cells are smaller and fewer in number (van Dijken *et al.*, 1975*a*; Tanaka *et al.*, 1976) and have no readily assignable specialized function, although in *Hansenula polymorpha* they are thought to develop into peroxisomes during growth on methanol (Veenhuis *et al.*, 1979) or on other substrates (Zwart *et al.*, 1983).

Despite a wealth of publications on activities, cytology and histochemistry of microbodies after growth on methanol (e.g. Tsubouchi *et al.*, 1976; Meisel *et al.*, 1978; Veenhuis *et al.*, 1981) there have been few reports on subcellular fractionation of methanol-grown yeasts, although enzymes involved with methanol dissimilation are thought to have different subcellular locations. After either sucrose or Ficoll density gradient fractionation of *Candida boidinii*, alcohol oxidase and catalase are recovered from heavy microbody-enriched fractions (Fukui *et al.*, 1975; Roggenkamp *et al.*, 1975). Differential centrifugation of cell extracts has shown that formaldehyde and formate dehydrogenases are probably soluble enzymes (Fukui *et al.*, 1975; Sahm *et al.*, 1975; Tanaka *et al.*, 1976). No systematic study has been made of the subcellular localization of the enzymes involved in the assimilation of methanol and there are no reports on subcellular fractionation of methylotrophic yeasts after growth on glucose.

This paper presents, for the first time, detailed high resolution fractionations using sucrose density gradients of cell extracts of *Candida boidinii*. In addition, the influence of growth substrate on the subcellular distribution of organelles in these gradients is discussed.

Abbreviation: DHA, dihydroxyacetone.

METHODS

Maintenance and growth. *Candida boidinii* CBS 2428 was maintained on potato dextrose agar slopes. The medium of Veenhuis *et al.* (1976) was used for growth, except that yeast extract was added to 0.025%. Glucose or methanol was added at 0.8% (w/v) or 1.0% (w/v), respectively. Cultures were grown and organisms counted as described previously (Jenkins *et al.*, 1983). Mean generation times in exponential phase of growth on glucose and methanol were 1.55 and 3.47 h, respectively; cell concentrations in stationary phase were 1.6×10^8 and 2.6×10^7 cells ml⁻¹, respectively. Cultures were harvested at 3.0×10^7 organisms ml⁻¹ for glucose-grown cells and 1.0×10^7 organisms ml⁻¹ for methanol-grown cells.

Harvesting, preparation and disruption of spheroplasts. All centrifugation procedures were carried out at 4 °C except during harvesting of cells, when a temperature of 30 °C was maintained. Organisms were harvested by centrifugation at 6000 *g* in a 6 × 250 ml rotor of an MSE 18 centrifuge. Spheroplasts were prepared using zymolyase (Kogoya Co., Tokyo, Japan), as described previously (Jenkins *et al.*, 1983). Preparation of cell extract was as described previously (Jenkins *et al.*, 1983) using a spheroplast disruption buffer containing 0.25 M-sucrose, 2 mM-MgCl₂ and 10 mM-Tris/HCl (pH 7.4). In studies involving assays for dihydroxyacetone (DHA) synthase, the disruption buffer also contained 0.5 mM-thiamin pyrophosphate, 1 mM-DTT and 0.024% phenylmethanesulphonyl fluoride. In some instances, disruption buffers containing 0.4 or 0.9 M-sucrose, sorbitol (0.25, 0.4, 0.65 or 0.9 M), or mannitol (0.25, 0.4 or 0.9 M) in place of 0.25 M-sucrose were used and centrifugation was adjusted to ensure removal of whole cells and intact spheroplasts.

Fractionation of cell extract by differential and zonal centrifugation. Differential centrifugations were carried out in an 8 × 50 ml rotor of an MSE 18 centrifuge at 20000 *g* for 20 min, as described previously (Jenkins *et al.*, 1983). Zonal centrifugations were carried out in a BX1 V aluminium rotor of an MSE 50 Superspeed centrifuge at 25000 *g* for 236 min. Fractionations through sucrose gradients were performed by the method of Cartledge & Lloyd (1972), except that for methanol-grown cells gradient construction included 40 ml of 52% (w/w) sucrose, and 0.5 mM-thiamin pyrophosphate, 1 mM-DTT and 0.024% phenylmethanesulphonyl fluoride were incorporated throughout the gradient.

Enzyme assays. The following assays were carried out by previously published methods: citrate synthase (EC 4.1.3.7) (Srere *et al.*, 1963); catalase (EC 1.11.1.6) (Luck, 1963); formaldehyde dehydrogenase (EC 1.2.1.1) (Rose & Racker, 1966); formate dehydrogenase (EC 1.2.1.2) (Quayle, 1966); NADPH:cytochrome *c* oxidoreductase (EC 1.6.2.4) (Duppel *et al.*, 1973) in the presence of 10 µg antimycin A; acid *p*-nitrophenyl phosphatase (EC 3.1.3.2) and alkaline *p*-nitrophenyl phosphatase (EC 3.1.3.1) (Torriani, 1960); methanol oxidase (EC 1.1.3.13) (Borman & Sahm, 1978). DHA synthase was assayed by two methods. (1) By measuring the xylulose 5-phosphate-dependent rate of removal of formaldehyde (discontinuous assay; Waites & Quayle, 1980). (2) By measuring the rate of NADH oxidation due to formaldehyde and xylulose 5-phosphate-dependent formation of triose phosphates (continuous assay; Waites & Quayle, 1981). 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 as described by Smith (1955), except that cytochrome *c* was previously reduced with dithionite and passed through a G25 Sephadex column. Inosine diphosphatase (EC 3.6.1.6) was assayed according to Delaissé *et al.* (1981), except that 2 mM-inosine 5'diphosphate was used.

Triton X-100 (final concentration 0.01%) was added to enzyme mixtures for cytochrome *c* oxidase, malate dehydrogenase and citrate synthase. Acid and alkaline phosphatase assays were carried out in the presence of 0.2% Triton X-100. Various concentrations of this reagent were shown not to increase the detectable activities of any of the remaining enzymes assayed and were therefore excluded from these enzyme assays. Inosine diphosphatase was assayed after rapid freezing and thawing of samples.

Preliminary experiments were carried out with cell extracts to ensure that the enzyme activities were proportional to the amount of extract protein used and were not limited by substrate concentration. Where necessary, appropriate dilutions of cell extracts and fractions were used to obtain a suitable enzyme rate.

Electron microscopy. Electron micrographs were prepared as described by Jenkins *et al.* (1983).

Analytical methods and chemicals. Protein was estimated by the Lowry method using bovine serum albumin as standard. Ingredients of enzyme assays were obtained from Sigma, except 2,2'-azino-di-[3-ethyl-benzthiazoline sulphonate (6)] and a mixture of triose phosphate isomerase and glycerol-3-phosphate dehydrogenase which were obtained from Boehringer. Formaldehyde was prepared from an aqueous suspension of paraformaldehyde by heating in a sealed ampoule at 110 °C for 20 h.

RESULTS

Differential centrifugation of cell extracts

Whereas cytochrome *c* oxidase activity was virtually all sedimentable in extracts from both glucose-grown and methanol-grown cells, high percentage sedimentabilities of malate dehydrogenase and catalase were obtained only for extracts of glucose-grown cells (Table 1).

Table 1. *Sedimentability of selected enzymes in cell extracts*

Sedimentability was calculated as a percentage of the total recovered activity present in the pellet formed following centrifugation at 20 000g for 20 min. The values presented are mean results of at least five experiments. Recoveries of enzyme activity were between 85 and 105%.

	Sedimentability (%)	
	Glucose-grown	Methanol-grown
Cytochrome <i>c</i> oxidase	100	98
Malate dehydrogenase	91	46
Catalase	70	29
Methanol oxidase	ND	80

ND, Not detectable.

Methanol oxidase activity, detectable only after growth on methanol, was largely sedimentable (Table 1).

Solubilization of catalase during incubation of methanol-grown cells with zymolyase was low, representing less than 4% of the total non-sedimentable catalase obtained following disruption of harvested spheroplasts in 0.2 M-potassium phosphate buffer, pH 7.4.

In an attempt to increase the percentage sedimentabilities of catalase and malate dehydrogenase in extracts from methanol-grown cells, alterations were made to the osmotic stabilizer in the spheroplast disruption buffer. Improved sedimentabilities of catalase and malate dehydrogenase (34 and 54%, respectively) were obtained only when using a disruption buffer containing 0.9 M-sorbitol in place of 0.25 M-sucrose. However, disruption of spheroplasts in 0.9 M-sorbitol was poor and activities of enzymes in these extracts were 70 to 80% lower than those in extracts prepared using 0.25 M-sucrose. Prolonged homogenization of spheroplasts in 0.9 M-sorbitol lowered the percentage sedimentabilities of catalase and malate dehydrogenase in cell extracts.

Zonal centrifugation of an extract from glucose-grown cells

Profiles of optical density at 260 nm and 520 nm showed major peaks at $\rho = 1.10 \text{ g ml}^{-1}$ and $\rho = 1.19 \text{ g ml}^{-1}$, respectively (Fig. 1a). Some protein was recovered at these densities but the majority (52%) remained in the region of the sample zone (Fig. 1a). This region also contained virtually all alkaline phosphatase activity (Fig. 1b), all formaldehyde dehydrogenase (Fig. 1d) and antimycin A-insensitive NADPH:cytochrome *c* oxidoreductase (Fig. 1e) activities and non-sedimentable activities of the other enzymes assayed. Acid phosphatase activity (Fig. 1b) was 32% sedimentable, located in a zone containing all cytochrome *c* oxidase (Fig. 1c) and greater than 90% of the activities of malate dehydrogenase and citrate synthase (Fig. 1c). Peak activities of enzymes in this zone were at $\rho = 1.19 \text{ g ml}^{-1}$, corresponding with maximum optical density at 520 nm. Sedimentable catalase activity (58%) was distributed broadly over densities of 1.15 to 1.25 g ml^{-1} (Fig. 1d), but the peak of this activity was found at a lower density ($\rho = 1.17 \text{ g ml}^{-1}$) than that of cytochrome *c* oxidase. The distribution of inosine diphosphatase (Fig. 1e) was complex, with most activity recovered over densities of $\rho = 1.07$ to 1.12 g ml^{-1} and remaining activity distributed between the sample zone and a zone at $\rho = 1.20 \text{ g ml}^{-1}$.

Activities of methanol oxidase and formate dehydrogenase were non-detectable in this cell extract.

Zonal centrifugation of an extract from methanol-grown cells

Sucrose gradient fractionation in a tube (23 ml) showed that sedimentable activities of cytochrome *c* oxidase and methanol oxidase are distributed mainly over densities of 1.20 to 1.27 g ml^{-1} (data not shown). In order to attempt to improve separation of organelles over these densities, a shallower region was incorporated into the gradient at a density of 1.24 g ml^{-1} and fractionation was carried out using the zonal rotor.

Fig. 1. Zonal centrifugation of a cell extract from glucose-grown *C. boidinii* on a sucrose gradient. Cell extract (10 ml) containing 63 mg of protein was loaded on the gradient. Centrifugation was at 28000 r.p.m. for 236 min (6×10^6 g-min at the sample zone: $\int_0^t \omega^2 \cdot dr = 1.45 \times 10^{11}$ rad² s⁻¹). Enzyme activities shown on the ordinates are mU ml⁻¹, where one unit represents 1 μ mol substrate used or product formed min⁻¹. Where indicated in the text percentage sedimentability refers to the amount of enzyme activity detected beyond $\rho = 1.10$ g ml⁻¹. (a) Sucrose density gradient (○), OD₅₂₀ (—) measured using an SP1800, OD₂₆₀ (---) and protein (●). (b) Alkaline phosphatase (○) and acid phosphatase (●). (c) Cytochrome *c* oxidase (○), malate dehydrogenase (●) and citrate synthase (□). (d) Catalase (○) and formaldehyde dehydrogenase (●). (e) Antimycin A-insensitive NADPH:cytochrome *c* oxidoreductase (○) and inosine diphosphatase (●). Specific activities [mU (mg protein)⁻¹] of enzymes in the cell extract were as follows (recoveries in parentheses): protein (115%); alkaline phosphatase, 87 (74%); acid phosphatase, 1.9 (108%); cytochrome *c* oxidase, 238 (68%); malate dehydrogenase, 3900 (101%); citrate synthase, 202 (98%); catalase, 11 (92%); formaldehyde dehydrogenase, 21 (78%); antimycin A-insensitive NADPH:cytochrome *c* oxidoreductase, 52 (62%); inosine diphosphatase, 26 (98%).

The main peak of optical density at 260 nm was located at $\rho = 1.07 \text{ g ml}^{-1}$ (Fig. 2a). Profile of optical density at 520 nm clearly defined two regions of the gradient: maximum OD at $\rho = 1.20 \text{ g ml}^{-1}$ and a further peak at $\rho = 1.27 \text{ g ml}^{-1}$ (Fig. 2a). Sedimentable protein (42%) was broadly distributed over densities of 1.15 to 1.27 g ml^{-1} (Fig. 2a). Non-sedimentable protein was almost entirely located in the sample zone. The region of the sample zone contained virtually all alkaline phosphatase activity (Fig. 2b) and all of the activities of antimycin A-insensitive NADPH:cytochrome *c* oxidoreductase (Fig. 2e) and formaldehyde and formate dehydrogenases (Fig. 2f). Most if not all of the non-sedimentable activities of the other enzymes assayed were also recovered from this region. The sedimentable portion of acid phosphatase activity (59%) was greater than that obtained for the extract from glucose-grown cells. The peak of this activity was located at a marginally higher density of $\rho = 1.20 \text{ g ml}^{-1}$ (Fig. 2b), corresponding with the maximum optical density at 520 nm. All cytochrome *c* oxidase and sedimentable citrate synthase (66%) formed almost identical symmetrical zones of activity (Fig. 2c) with peak activities at $\rho = 1.20 \text{ g ml}^{-1}$. Sedimentable activities of malate dehydrogenase (48%; Fig. 2c) and DHA synthase (23 to 33%; Fig. 2e) were also recovered from this region. An electron micrograph of this region of the gradient (Fig. 3a) shows the presence of mitochondria and small membraneous vesicles. Similar distributions of the latter enzyme were obtained by continuous and discontinuous assays and satisfactory recoveries of activity were obtained. An electron micrograph of the region at $\rho = 1.23 \text{ g ml}^{-1}$ (Fig. 3b) indicates the presence of mitochondria, microbodies and membraneous vesicles while the region at $\rho = 1.27 \text{ g ml}^{-1}$ contains microbodies and little other membraneous material (Fig. 3c). Sedimentable activities of catalase (19%) and methanol oxidase (88%) formed similar bimodal distributions (Fig. 2d), with distinct peaks of activity located at $\rho = 1.23 \text{ g ml}^{-1}$ and 1.27 g ml^{-1} , the latter corresponding with a maximum optical density at 520 nm. Inosine diphosphatase (Fig. 2e) was recovered largely from the sample zone although a peak of activity at $\rho = 1.07 \text{ g ml}^{-1}$, corresponded with the maximum of optical density at 260 nm. Sedimentable activities of this enzyme (30%) showed a broad density distribution with a small peak at $\rho = 1.23 \text{ g ml}^{-1}$.

DISCUSSION

The oxidative metabolism of methanol to CO_2 is indicated by the high specific activities of methanol oxidase, catalase, formaldehyde dehydrogenase and formate dehydrogenase in extracts from methanol-grown cells. The comparatively low or non-detectable activities of these enzymes in extracts from glucose-grown cells probably reflects control of their synthesis by catabolite repression–derepression (Egli *et al.*, 1980).

Decreased sedimentability of catalase and possibly malate dehydrogenase in extracts from methanol-grown relative to glucose-grown cells indicates increased fragility of organelles. This hypothesis is supported by cytochemical evidence that catalase is located solely within microbodies of methanol-grown yeast (van Dijken *et al.*, 1975b; unpublished data from our laboratory). Gross solubilization of catalase during spheroplast formation was not apparent and changes made to the osmolarity of the spheroplast disruption buffer had little effect on sedimentability of enzymes. The results of Fukui *et al.* (1975) show that the activities of both catalase and methanol oxidase are largely non-sedimentable after disruption of spheroplasts of methanol-grown *C. boidinii* in 0.65 M-sorbitol. Our results show that in 0.25 M-sucrose, high percentage sedimentability of methanol oxidase but not catalase can be achieved. The organization of these enzymes within microbodies (Veenhuis *et al.*, 1981) probably accounts for their difference in sedimentability: whereas catalase was shown to be largely a matrix enzyme, methanol oxidase was a structural element of the crystalline core.

After fractionation of extracts from either glucose or methanol-grown cells a number of distinct zones on sucrose gradients can be characterized.

Non-sedimentable enzyme activities recovered from the proximity of the sample zone may reflect a truly cytosolic location for the enzymes and/or loss of enzymes from membraneous organelles. The presence of all recovered activities of formate and formaldehyde dehydrogenases and antimycin A-insensitive NADPH:cytochrome *c* oxidoreductase in this region favours

Fig. 2. Zonal centrifugation of a cell extract from methanol-grown *C. boidinii* on a sucrose gradient. Cell extract (10 ml) containing 78 mg of protein was loaded on the gradient. Centrifugation was at 28000 r.p.m. for 236 min ($6 \times 10^6 \text{ g} \cdot \text{min}$ at the sample zone; $\int_0^t \omega^2 \cdot dt = 1.45 \times 10^{11} \text{ rad}^2 \text{ s}^{-1}$). Enzyme activities shown on the ordinates are mU ml^{-1} , where one unit represents $1 \mu\text{mol}$ substrate used or product formed min^{-1} . Where indicated in the text percentage sedimentability refers to the amount of

the first possibility. A cytosolic localization for the first two enzymes confirms the findings of other workers (Fukui *et al.*, 1975; Sahm *et al.*, 1975; Tanaka *et al.*, 1976) and for the last enzyme indicates that it is not a suitable microsomal marker for this organism as suggested by Cartledge & Lloyd (1972) in *Saccharomyces carlsbergensis*.

The mitochondrial zone, characterized by cytochrome *c* oxidase activity, was readily identified and contained all the sedimentable activities of malate dehydrogenase and citrate synthase. Increased sedimentable activities of citrate synthase, and possibly malate dehydrogenase, after fractionation of methanol-grown cells relative to glucose-grown cells presumably indicates differences in fragility of mitochondria. For both fractionations the sedimentable activity of acid phosphatase, an enzyme characteristically associated with yeast vacuoles, was also recovered from the mitochondrial zone. After similar fractionations using metrizamide gradients this activity was well separated from cytochrome *c* oxidase activity (R. O. Jenkins, unpublished results).

After fractionation of glucose-grown cells, the microbody region, as defined by sedimentable catalase activity, was found at a lower density ($\rho = 1.17 \text{ g ml}^{-1}$) than the region containing mitochondria. After fractionation of methanol-grown *C. boidinii*, Fukui *et al.* (1975) reported microbodies as heavier than mitochondria in sucrose. Whilst our results agree with this finding they also show that after growth on methanol two microbody zones at densities of 1.23 and 1.27 g ml^{-1} can be distinguished using a zonal rotor, as opposed to the single broad density region distinguished by these workers using a small volume (15 ml) tube gradient. The localization of methanol oxidase within these zones (both of which contain intact microbodies, as confirmed electron microscopically) allows the term peroxisome, defined by Tolbert & Essner (1981), to be assigned to associated organelles. Whether the observed heterogeneity represents two functionally distinct classes of peroxisomes in *C. boidinii* or arises from damage during isolation and separation requires further investigation.

Activity of DHA synthase, a key methanol assimilation enzyme (O'Connor & Quayle, 1980; Waites & Quayle, 1980), was partially sedimentable and was recovered in the fraction containing mitochondria and membraneous vesicles. Sedimentable activity is difficult to explain as other enzymes involved in methanol assimilation, for example fructose-1,6-bisphosphatase, have been shown cytochemically to be in the cytosol (Veenhuis *et al.*, 1980). Both continuous and discontinuous assays for DHA synthase gave very similar results and all relevant precautions (Waites & Quayle, 1980, 1981) to measure only DHA synthase activity and eliminate any enzymes such as transketolase, alcohol dehydrogenases and formaldehyde oxidase were carefully carried out.

Inosine diphosphatase appears to be multilocational in these cells. The recovery of large portions of this enzyme's activity at low densities distinct from the sample zone indicates that the occurrence of inosine diphosphatase in unidentified small membraneous vesicles, as reported by Delaissé *et al.* (1981) for *Saccharomycopsis lipolytica*, must be considered.

The results presented here show that the distribution of organelles on sucrose gradient can be markedly influenced by the nature of the growth substrate. After growth on methanol, a

enzyme activity detected beyond $\rho = 1.10 \text{ g ml}^{-1}$. (a) Sucrose density gradient ($\bigcirc$), OD_{520} (—) measured using an SP1800, OD_{260} (---), protein ($\bullet$). (b) Alkaline phosphatase ($\bigcirc$) and acid phosphatase ($\bullet$). (c) Cytochrome *c* oxidase ($\bigcirc$), malate dehydrogenase ($\bullet$) and citrate synthase ($\square$). (d) Catalase ($\bigcirc$), catalase (sedimented activity only; $\bigcirc$ --- $\bigcirc$) and methanol oxidase ($\bullet$). (e) DHA synthase (continuous assay; $\bigcirc$), DHA synthase (discontinuous assay; $\bullet$) and antimycin A-insensitive NADPH:cytochrome *c* oxidoreductase ($\square$). (f) Formate dehydrogenase ($\bigcirc$), formaldehyde dehydrogenase ($\bullet$) and inosine diphosphatase ($\square$). Specific activity [$\text{mU}(\text{mg protein})^{-1}$] of enzymes in the cell extract were as follows (recoveries in parentheses): protein (107%); alkaline phosphatase, 12 (105%); acid phosphatase, 2 (68%); cytochrome *c* oxidase, 97 (117%); malate dehydrogenase, 12860 (98%); citrate synthase, 65 (89%); catalase, 974 (65%); methanol oxidase, 207 (121%); DHA synthase (continuous assay), 27 (103%); DHA synthase (discontinuous assay), 35 (96%); antimycin A-insensitive NADPH:cytochrome *c* oxidoreductase, 52 (78%); formate dehydrogenase, 142 (96%); formaldehyde dehydrogenase, 325 (91%); inosine diphosphatase, 26 (92%).

Fig. 3. Electron micrographs of section of material from experiment shown in Fig. 2. (a) Fraction 29, (b) fraction 32, (c) fraction 43. Bar markers, 0.5 μ m.

heterogeneous population of microbodies may be present in the cell, although possible diversity of function has yet to be established. Further work is also required to clarify the localization of enzymes involved in methanol assimilation.

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