Changes in proteinase activities and subcellular distribution during inactivation of alcohol oxidase in *Candida boidinii*

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1. Adaptation of methanol-grown *Candida boidinii* to ethanol utilization was accompanied by an increase in proteolytic activities, which behaved like known vacuolar enzymes. 2. Degradation of alcohol oxidase protein was partially prevented by the serine proteinase inhibitor phenylmethanesulphonyl fluoride, but not by the carboxyl proteinase inhibitor pepstatin. 3. Fractionation of cell-free extracts, by high-speed zonal centrifugation, of methanol-grown *C. boidinii* showed non-sedimentable and sedimentable proteolytic activities. Naturally occurring inhibitors of vacuolar proteinases were non-sedimentable. 4. Fractionation of extracts prepared from methanol-grown cells which had been adapted to ethanol utilization for 5 h revealed significant changes in the sedimentability and distribution of proteolytic and acid phosphatase activities. 5. These results suggest the possible involvement of a vacuolar process during alcohol oxidase degradation.

INTRODUCTION

Selective inactivation of enzymes, defined as the irreversible loss *in vivo* of catalytic activity, has been recognized as an important mechanism for the control of enzyme activity. Two distinct inactivation processes have been observed: (a) modification inactivation, in which the enzyme protein remains intact but loses activity through a change in its physical state or the attachment of a covalent modifying group, and (b) degradative inactivation, in which at least one peptide bond of the protein is cleaved (Switzer, 1977). Protein hydrolysis may, or may not, follow the initial inactivation process.

Inactivation of enzymes occurs when organisms are exposed to an altered environment, leading to conditions which no longer require the activity of those proteins for growth. Glucose-induced inactivations have been observed for a variety of enzymes, of which fructose-1,6-bisphosphatase has been the most extensively studied. This phenomenon was termed 'catabolite inactivation' by Holzer (1976), who proposed a proteolytic mechanism for the degradative inactivation of certain enzymes. The proteolytic system in yeasts, reviewed by Wolf (1980), has been shown to comprise many different proteinases, of varied intracellular localization. It is well established in *Saccharomyces cerevisiae* that the main endoproteolytic activity is located in the vacuole, and that naturally occurring inhibitors of these vacuolar endoproteinases exist within the cytosol (Lenney *et al.*, 1974; Matern *et al.*, 1974). A vacuolar process was therefore envisaged during degradative inactivations in yeasts.

The growth of *Candida boidinii* with methanol, but not with ethanol, is associated with the induction of large numbers of peroxisomes mainly containing catalase and the H₂O₂-producing alcohol oxidase (Roggenkamp *et al.*, 1975). It has been demonstrated that in methanol-grown *C. boidinii* the enzyme alcohol oxidase is degradatively inactivated upon changing the surrounding conditions to

one which supports an energetically more favourable metabolic pathway, namely ethanol utilization (Hill *et al.*, 1985). Similar results have been obtained from the glucose-induced inactivation of methanol-grown *Hansenula polymorpha*, in which a vacuolar process was proposed (Veenhuis *et al.*, 1983).

In the present study we discuss the involvement of vacuolar-like proteinases during the adaptation of methanol-grown *C. boidinii* to ethanol and provide further evidence for the involvement of the vacuole during degradative inactivation of alcohol oxidase.

METHODS AND MATERIALS

Micro-organism, culture conditions, and adaptation to ethanol utilization

C. boidinii CBS5777 was grown at 30 °C with shaking in a mineral-salts medium containing 124 mM-methanol as the sole source of carbon and energy as described previously (Haywood & Large, 1981). For adaptation to ethanol utilization, organisms were harvested at 30 °C in the late-exponential phase of growth, transferred into 100 mM-potassium phosphate buffer (pH 6.0) containing 174 mM-ethanol, and re-incubated with shaking at 30 °C. Cell growth was measured by the increase in the *A*₆₆₃.

Harvesting and breakage of the organism

Cells were harvested at 4 °C, in a 6 × 250 ml rotor of an MSE 18 centrifuge at 8 × 10⁴ g · min. Pellets were washed twice by resuspension in 50 mM-potassium phosphate buffer (pH 7.2).

Breakage was achieved with a Braun disintegrator (F. T. Scientific, Tewkesbury, Glos., U.K.) operating at 4000 Hz for 4 min with liquid-CO₂ cooling. Whole cells were removed by centrifugation at 8 × 10⁴ g · min and the supernatant was used without further fractionation.

Abbreviation used: PMSF, phenylmethanesulphonyl fluoride.

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Fig. 1. Changes in proteolytic activities during adaptation of methanol-grown cells to ethanol utilization

(a) Changes in 'unactivated' proteolytic activity of cell-free extracts during adaptation of methanol-grown cells to ethanol: ●, alcohol oxidase activity; proteolytic activity (■) at pH 3.0 and (□) at pH 6.3; △, A_{663} of cell-free suspension. (b) Changes in 'activated' proteolytic activity of cell-free extracts during adaptation of methanol-grown cells to ethanol; symbols as in (a). (c) Control experiment showing changes in alcohol oxidase activity (●) and 'activated' proteolytic activity at pH 3.0 (■) and at pH 6.3 (□), in extracts from methanol-grown cells incubated aerobically in potassium phosphate buffer (pH 6.0) in the absence of ethanol.

Preparation and disruption of sphaeroplasts

Sphaeroplasts 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), with a sphaeroplast-disruption buffer containing 0.25 mM-sucrose, 2 mM-MgCl₂ and 10 mM-Tris/HCl (pH 7.4).

Enzyme assays

Alcohol oxidase (EC 1.1.3.13) activity in whole cells and extracts was determined polarographically as described by van Dijken *et al.* (1976), except in the subcellular-fractionation experiments, where it was determined as described by Bormann & Sahm (1978), by the dye [2,2-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid)]-linked assay. Catalase (EC 1.11.1.6) was assayed as described by Luck (1963). 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 Sephadex G-25 column. Alkaline *p*-nitrophenyl phosphatase (EC 3.1.3.1) and acid *p*-nitrophenyl phosphatase (EC 3.1.3.2) were assayed as described by Torriani (1960).

Triton X-100 (final concn. 0.01%) was added to the cytochrome *c* oxidase assay mixture. Acid and alkaline phosphatase assays were carried out in the presence of 0.2% Triton X-100.

Spectrophotometric assays were carried out with a Pye-Unicam SP.1800 u.v. spectrophotometer. All assays were carried out at 30 °C.

Proteolytic activity was determined by casein hydrolysis as follows: 50 µl of sample was incubated with 100 µl of [³H]casein solution (10 mg/ml; 2.5 × 10⁶ d.p.m./mg of casein) at the defined pH, for up to 1 h at 30 °C. The reaction was stopped with 250 µl of ice-cold trichloroacetic acid (11%, v/v) and kept in an ice bath for 30 min. The precipitated casein was removed by centrifugation at 11000 *g* for 1 min. Then 100 µl of the supernatant was

Fig. 2. Changes in proteolytic activities over 15 h of adaptation of methanol-grown cells to ethanol

Proteolytic activities in extracts prepared from methanol-grown cells undergoing adaptation to ethanol were determined: 'unactivated' (---) and 'activated' (—) proteolytic activity (■) at pH 3.0 and (□) at pH 6.3.

removed into 5 ml of scintillation fluid (Aqualuma Plus; Lumac Systems, Titusville, FL, U.S.A.) and the radioactivity counted in a Beckman LS230 scintillation counter. Samples were counted for 1 h and results corrected for quenching. Background radioactivity was determined in the absence of enzyme and represented less than 1% of the original activity. Counting efficiency was approx. 30%.

Activation of proteinases in cell extracts and subcellular fractions was performed by the method of Saheki & Holzer (1975), by incubation at pH 5.1 at 25 °C in order to hydrolyse the inhibitors from the inactive proteinase complexes. Incubation mixtures contained (per ml of extract): 50 μ l of 10% acetic acid, 50 μ l of 0.05 M-acetate buffer, pH 5.1, and 1 μ l of antibiotic mixture. The antibiotic mixture contained (per ml): streptomycin sulphate, 50 mg; penicillin G, 40 000 i.u.; nystatin, 10 000 i.u.; neomycin sulphate 10 mg (Hansen *et al.*, 1977). Proteolytic activities are expressed as μ g of casein hydrolysed/min per mg of protein.

Determination of alcohol oxidase protein

Polyacrylamide-gel electrophoresis was used to quantify alcohol oxidase protein in cell extracts as described previously (Hill *et al.*, 1985).

Fractionation of cell extract by zonal centrifugation

Zonal centrifugations were carried out in a BXIV aluminium rotor of an MSE 50 Superspeed centrifuge at 25 000 *g* for 236 min. Fractionations were carried out as described by Jenkins *et al.* (1985) on sucrose gradients.

Protein was determined by the method of Lowry *et al.* (1951).

RESULTS

Involvement of proteinases during adaptation of methanol-grown *C. boidinii* to ethanol utilization

During adaptation of methanol-grown *C. boidinii* to ethanol metabolism, in which peroxisomes and the peroxisomal enzyme alcohol oxidase are degraded, only a small increase in proteolytic activity was detectable (Fig. 1a) in fresh ('unactivated') extracts (i.e. in the presence of naturally occurring proteinase inhibitors) at pH 3.0 or at pH 6.3. However, after 'activation' of the extracts, a process during which proteinase inhibitors were removed, a several-fold increase in proteolytic activity was observed (Fig. 1b) for casein hydrolysis at pH 3.0, and an even greater increase at pH 6.3. Incubation of methanol-grown cells in potassium phosphate buffer (pH 6.0), conditions which gave no degradation of alcohol oxidase (Hill *et al.*, 1985), revealed that proteolytic activities, in activated extracts, showed activities that did not significantly change during 7 h of incubation (Fig. 1c).

In longer experiments in which methanol-grown cells were incubated in the presence of ethanol, maxima for 'activated' proteolytic activities were observed after 8–11 h adaptation; subsequently activities observed at both pH 3.0 and 6.3 declined (Fig. 2).

Proteinases increasing during adaptation to ethanol were characterized by their sensitivity towards inhibitors (Table 1). In 'activated' extracts prepared from cells growing exponentially with methanol, the proteolytic activity was inhibited by up to 60% and 70% by

Table 1. Characterization of proteinases of 'activated' extracts before and after ethanol inactivation

Results are expressed with 95% confidence limits determined from quadruplicate assays, and are for extracts from methanol-grown cells (a) before and (b) after 7 h ethanol inactivation.

	Inhibitor	Remaining proteolytic activity (%) assayed at:	
		pH 3.0	pH 6.3
(a)	None	100	100
	Pepstatin (1.46 μ M)	40.4 $\pm$ 9.2	31.4 $\pm$ 9.7
	PMSF (2 mM)	75.7 $\pm$ 21.6	65.2 $\pm$ 23.1
	EDTA (2 mM)	114.1 $\pm$ 20.1	91.0 $\pm$ 26.3
	Iodoacetate (2 mM)	83.7 $\pm$ 27.3	90.2 $\pm$ 60.0
(b)	None	100	100
	Pepstatin (1.46 μ M)	70.1 $\pm$ 2.1	69.6 $\pm$ 6.1
	PMSF (2 mM)	30.3 $\pm$ 1.8	30.2 $\pm$ 2.8
	EDTA (2 mM)	49.6 $\pm$ 2.1	87.5 $\pm$ 6.1
	Iodoacetate (2 mM)	90.0 $\pm$ 1.6	91.4 $\pm$ 7.2

pepstatin and by up to 25% and 35% by PMSF at pH 3.0 and pH 6.3 respectively. There was no significant detectable inhibition of the proteolytic activity by the inhibitors EDTA or iodoacetate.

'Activated' extracts from methanol-grown cells after 7 h adaptation to ethanol gave increased proteolytic activities which were inhibited by pepstatin by up to 30%, and by up to 70% by PMSF, at both pH 3.0 and pH 6.3. These results, compared with methanol-grown cells, represent a 3–3.5-fold increase in pepstatin-sensitive proteolytic activity and a 14–21-fold increase in PMSF-sensitive proteolytic activity. The extracts prepared from ethanol-adapted cells also contained proteinases sensitive to EDTA and iodoacetate, but these represented less than 10% of the total activity.

The effect of PMSF on the respiration of methanol during ethanol adaptation was studied at various concentrations of this serine proteinase inhibitor. Increasing concentrations of PMSF resulted in decreasing rates of inactivation of alcohol oxidase activity in whole cells (Fig. 3a). Pepstatin (2 μ M) showed no inhibition of alcohol oxidase activity in whole cells.

Alcohol oxidase protein contents were determined, by gel electrophoresis, for samples prepared from cells which had been ethanol-adapted for 6 h in the presence of various concentrations of inhibitors, and the results expressed as percentages of those in methanol-grown cells. Increasing concentrations of PMSF resulted in an increasing content of alcohol oxidase protein, which was reflected in the increase in alcohol oxidase activity (Fig. 3a). Pepstatin did not inhibit the decrease in methanol-supported respiration of whole cells during ethanol adaptation, and this was confirmed by the results shown in Fig. 3(b), which showed that increasing pepstatin concentrations did not affect the content of alcohol oxidase protein, and hence did not inhibit enzyme degradation.

Ethanol inactivation of methanol-grown cells in the presence of chloroquine, an inhibitor which accumulates in the vacuole and inhibits vacuolar proteinases (Lenz & Holzer, 1984), showed that the decrease in alcohol

Fig. 3. Effects of (a) PMSF and (b) pepstatin on alcohol oxidase activity of whole cells and alcohol oxidase protein in extracts during ethanol adaptation

Alcohol oxidase protein was determined by gel electrophoresis of cell-free extracts prepared from methanol-grown cells which had been ethanol-adapted for 6 h in the presence of various concentrations of inhibitors. Alcohol oxidase activity was determined in whole cells, polarographically. Results are expressed as percentages of original values determined for methanol-grown cells. Effects of (a) PMSF and (b) pepstatin on alcohol oxidase activity (●) and alcohol oxidase protein determined by polyacrylamide-gel electrophoresis (□) or SDS/polyacrylamide-gel electrophoresis (■).

oxidase activity in whole cells was partly prevented by 50 mM-chloroquine (Fig. 4).

High-speed zonal fractionation of cell-free extracts on sucrose gradients

Fractionations of extracts prepared with Zymolyase were carried out with a BXIV zonal rotor.

(a) Extract from methanol-grown cells. After centrifugation, approx. 50% of the protein remained in the sample zone (Fig. 5a). Acid and alkaline phosphatases were 38% and 10% sedimentable respectively, being recovered at densities (g/ml) of 1.15–1.30 and 1.20–1.30, with maxima

Fig. 4. Effect of chloroquine on alcohol oxidase during ethanol adaptation of methanol-grown *C. boidinii*

Methanol-grown cells were washed and resuspended in potassium phosphate buffer (pH 6.0) containing chloroquine at 0–50 mM final concn. After 30 min incubation, ethanol was added to a final concentration of 174 mM. Alcohol oxidase activity was determined for washed whole-cell suspensions by oxygen uptake in the presence of 33 mM-methanol after 8 h: 100% represents alcohol oxidase activity at time of ethanol addition.

at 1.22 and 1.23 respectively (Fig. 5b). Sedimentable activities of catalase (51.5%) and methanol oxidase (66.2%) showed similar distribution patterns, being recovered at densities of 1.15–1.28, with maxima at 1.24 (Fig. 5c). All cytochrome *c* oxidase activity (Fig. 5d) was sedimentable, and peak activities were recovered at a density of 1.21. Proteolytic activities were determined at pH 3.0, 6.3 and 9.2. Proteolytic activity at pH 3.0 was 31% and 24.5% sedimentable for 'unactivated' and 'activated' samples respectively, and showed a broad distribution over a density range of 1.10–1.30 (Fig. 5e). At pH 6.3 proteolytic activity was 59.5% and 56.8% sedimentable for 'unactivated' and 'activated' activities, being recovered over a broad density range of 1.10–1.35, with maximal activity at 1.23. Distribution of proteolytic activity at pH 6.3 after activation showed little activity up to a density of 1.18, with most activity sedimenting above this density, but at lower activities than for the 'unactivated' sample (Fig. 5f). Proteolytic activity determined at pH 9.2 was 40.7% and 40.3% sedimentable for 'unactivated' and 'activated' activities respectively. The sedimentable 'unactivated' activity at pH 9.2 was distributed over a broad density range of 1.10–1.30, with a peak activity at 1.23. Activation of samples resulted in a decrease in sedimentable activities (Fig. 5g). Activation of proteinase only gave increased activities in the sample zone for proteolytic activity determined at pH 3.0, 6.3 and 9.2.

(b) Ethanol-adapted cells. Fractionation of an extract prepared from methanol-grown cells adapted to ethanol for 4 h was done by a procedure identical with that used for methanol-grown cells.

After centrifugation, 57% of the protein remained in the sample zone (Fig. 6a). Acid phosphatase activity showed 67% sedimentability (Fig. 6b), which was recovered over a broad density range of 1.10–1.30 g/ml, with activity maxima at 1.20 and 1.23. Alkaline phosphatase activity showed 23% sedimentability, which was recovered as a broad peak over the density range

1.16–1.27, maximal around 1.23. Methanol oxidase activity was not detectable (< 0.05 nmol/min per mg) either in the extract or in any fraction. Catalase (Fig. 6c) showed 28% sedimentability, which was recovered over a broad density range of 1.16–1.27, maximal around 1.23. Cytochrome *c* oxidase activity was completely sedimentable, with maximal activity at a density of 1.20 (Fig. 6d).

Proteolytic activities were determined as previously. Casein hydrolysis at pH 3.0 showed 42% and 39.5% sedimentability (Fig. 6c) for 'unactivated' and 'activated' samples respectively. The sedimentable 'unactivated' activity, determined at pH 3.0, was distributed throughout the density gradient, with definite maxima at 1.13 and 1.22, whereas 'activation' resulted in decreased maxima at 1.13 and 1.18. Proteolytic activity determined at pH 6.3 showed 59% sedimentability, which decreased to 55.9% after 'activation'. The sedimentable 'unactivated' activity revealed a maximum at density 1.13 and a broad maximum at 1.18–1.23; activation of the samples resulted in similar maxima at lower activities (Fig. 6f). Proteolytic activity determined at pH 9.2 showed 71% sedimentability, showing a general increase in sedimentable activity with increasing density, with maxima at 1.105, 1.15, 1.30 and 1.24; 'activation' resulted in substantially decreased activities at these maxima (Fig. 6g).

Activation of proteolytic activity was only observed in the sample zone for casein hydrolysis at pH 3.0, 6.3 and 9.2.

DISCUSSION

Adaptation of methanol-grown *C. boidinii* to ethanol utilization involves degradation of the methanol-induced peroxisomal enzyme alcohol oxidase and loss of peroxisomes (Hill *et al.*, 1985).

Naturally occurring inhibitors have only been demonstrated for vacuolar proteinases (Wolf, 1980). The

Fig. 5. High-speed zonal fractionation of an extract of methanol-grown cells on a sucrose gradient

Cell extract (10 ml, containing 87 mg of protein) from sphaeroplasts prepared by using Zymolyase was loaded on the gradient. Centrifugation in the BXIV zonal rotor was at 28000 rev/min for 236 min (6×10^6 g·min at the sample zone; $\int_0^t \omega^2 \cdot dt = 1.2 \times 10^{11} \text{ rad}^2 \cdot \text{s}^{-1}$). (a) sucrose density gradient (○); protein (●). (b) Alkaline phosphatase (○); acid phosphatase (●). (c) Catalase (○); alcohol oxidase (●). (d) Cytochrome *c* oxidase (○). (e) Proteolytic activity at pH 3.0: 'unactivated' (○) and 'activated' (●). (f) Proteolytic activity at pH 6.3: 'unactivated' (○) and 'activated' (●). (g) Proteolytic activity at pH 9.2: 'unactivated' (○) and 'activated' (●). The specific activities of enzymes in the cell extract were as follows (recoveries in parentheses): protein (113%); alkaline phosphatase 21.4 (40%); acid phosphatase 8.6 (130%); catalase 696 (95.7%); alcohol oxidase 78.1 (152.6%); cytochrome *c* oxidase 600 (46%); 'unactivated' proteolytic activities at pH 3.0, 3.31 (286%), at pH 6.3, 12.38 (535%), and at pH 9.2, 6.44 (985%); 'activated' proteolytic activities at pH 3.0, 5.36 (223%), at pH 6.3, 20.6 (337%), and at pH 9.2, 18.87 (340%). 'Activation' of proteinases was carried out after subcellular fractionation.

process of 'activation' has been shown to hydrolyse these inhibitors (Saheki & Holzer, 1975). That no increase in proteolytic activity in non-activated extracts occurs during adaptation of methanol-grown cells to ethanol, whereas activated proteolytic activity does (Fig. 1), suggests a specific effect on vacuolar proteinases during the adaptation process. It is possible that inhibitors of non-vacuolar proteinases may exist, but these have not been shown. Characterization of the proteinases involved during the adaptation revealed that the proteolytic activity, as determined by casein hydrolysis, was predominantly due to serine and carboxyl proteinases (increases in activity of 21-fold and 3-fold respectively). Studies of the effect of the inhibitors PMSF and pepstatin on the inactivation of alcohol oxidase *in vivo* showed that inactivation was partially prevented by the serine proteinase inhibitor PMSF, but not by the carboxyl proteinase inhibitor pepstatin. Inhibition of catabolite inactivation *in vivo* by PMSF has also been shown by Magni *et al.* (1978) and Mazon (1978). However, PMSF is not solely specific inhibitor of serine proteinases, but will inhibit any enzyme with a serine group at its active centre. Also, in the absence of inhibition by pepstatin we cannot rule out the possibility that the inhibitor did not reach its target site in an active form.

The increase in 'activated' proteolytic activity occurred several hours after the onset of inactivation of alcohol oxidase, at which time much of the alcohol oxidase was undetectable. This suggests that the increase in proteolytic activity observed during the adaptation was not responsible for the initial degradation of alcohol oxidase. In *S. cerevisiae*, studies have also been shown that mutations leading to the loss of the carboxyl proteinase A and/or the serine proteinase B, both vacuolar proteinases, did not affect the catabolite inactivation *in vivo* of the gluconeogenic enzymes fructose-1,6-bisphosphatase, malate dehydrogenase and phosphoenolpyruvate carboxykinase (Wolf, 1982).

Fig. 6. High-speed zonal fraction of an extract of 5 h ethanol-adapted methanol-grown cells on a sucrose gradient

Cell-free extract (10 ml, containing 32 mg of protein) was loaded on the gradient. Centrifugation in the BXIV zonal rotor was at 38000 rev./min for 236 min

($6 \times 10^6 g \cdot \text{min}$ at the sample zone; $\int_0^t \omega^2 \cdot dt = 1.2 \times 10^{11} \text{ rad}^2 \cdot \text{s}^{-1}$). (a) Sucrose density gradient (○); light-scattering at 260 nm (△); protein (●). (b) Alkaline phosphatase (○); acid phosphatase (●). (c) Catalase (○); alcohol oxidase (●). (d) Cytochrome *c* oxidase (○). (e) Proteolytic activity at pH 3.0: 'unactivated' (○) and 'activated' (●). (f) Proteolytic activity at pH 6.3: 'unactivated' (○) and 'activated' (●). (g) Proteolytic activity at pH 9.2: 'unactivated' (○) and 'activated' (●). The specific activities of enzymes in the cell extract were as follows (recoveries in parentheses): protein (105%); alkaline phosphatase 103 (79%); acid phosphatase 9.05 (89%); catalase 133.7 (92.6%); alcohol oxidase < 0.05 (-); cytochrome *c* oxidase 129 (88.5%); 'unactivated' proteolytic activities at pH 3.0, 11.14 (201%), at pH 6.3, 10.32 (285%), and at pH 9.2, 9.01 (388%); 'activated' proteolytic activities at pH 3.0, 3.7 (194%), at pH 6.3, 14.52 (188%), and at pH 9.2, 13.19 (260%). 'Activation' of proteinases was carried out after subcellular fractionation.

As the adaptation to ethanol occurred in the absence of an added nitrogen source, the observed increase in 'activated' proteolytic activity may be correlated with hydrolysis of the primary degradative products of alcohol oxidase to amino acids for recycling. Similar changes in proteolytic activity have been observed for *S. cerevisiae* at the onset of stationary phase or during nitrogen limitation (Hansen *et al.*, 1977; Lenney *et al.*, 1974).

Fractionation of extracts of methanol-grown cells and methanol-grown cells that had undergone adaptation to ethanol for 5 h revealed a number of distinct zones, which can be readily characterized. Non-sedimentable enzyme activities recovered from the proximity of the sample zone may reflect a truly cytosolic location for an enzyme and/or loss of enzymes from membraneous organelles. The mitochondrial zone is characterized by cytochrome *c* oxidase. Sedimentable acid phosphatase activity is characteristically associated with yeast vacuoles. Peroxisomes are characterized by sedimentable catalase and, in the case of methanol-grown cells, alcohol oxidase (Roggkamp *et al.*, 1975; Jenkins *et al.*, 1985).

Sedimentable proteolytic activity showed definite zones of activity, especially from cell extracts prepared from adapted cells. The high 'unactivated' proteolytic activity observed in the sample zone is unlikely to be due to leakage from vacuoles, as vacuolar proteinases would bind with their respective cytosolic endogenous inhibitors on release from the vacuole (Lenney *et al.*, 1974). Therefore the non-sedimentable endoproteinases predominantly represent cytosolic activities. Hydrolysis of the endogenous inhibitors by 'activation' gave increased proteolytic activities only in the sample zone, and this probably reflects activity attributable to leakage of vacuolar proteinases. The absence of increased activity of sedimentable proteinases after activation reflects the cytosolic localization of the endogenous proteinase inhibitors.

Ethanol-adapted cells were characterized by an absence of alcohol oxidase activity and lower activity and sedimentability of catalase. Significant changes of enzyme distribution as compared with unadapted cells were observed. Acid phosphatase gave increased sedimentability and a broader distribution of activity, along with the appearance of a major peak sedimenting at a higher density (identical with the peak density for sedimentable catalase). Increased sedimentability was also observed for proteolytic activities at pH 3.0 and pH 9.2 for adapted cells. Decreased activity of cytochrome *c* oxidase and a 5-fold increase in alkaline phosphatase

activity were also observed. Acid phosphatase and proteolytic activities showed no significant change in activity in adapted cells as compared with unadapted cells.

These results suggest that the degradation of alcohol oxidase and peroxisomes involves a vacuolar process. The observation of inhibition of inactivation of alcohol oxidase by chloroquine, an inhibitor of vacuolar enzyme activities (Lenz & Holzer, 1984), together with evidence from electron microscopy (Hill *et al.*, 1985), supports this hypothesis. The role of proteinases in the degradation of alcohol oxidase, however, remains unclear, and further work is required to elucidate their involvement.

REFERENCES

- Bormann, C. & Sahm, H. (1978) *Arch. Microbiol.* **117**, 67–72
 Hansen, R. J., Switzer, R. L., Hinze, H. & Holzer, H. (1977) *Biochim. Biophys. Acta* **496**, 103–114
 Haywood, G. W. & Large, P. J. (1981) *Biochem. J.* **199**, 187–201
 Hill, D. J., Hann, A. C. & Lloyd, D. (1985) *Biochem. J.* **232**, 743–750
 Holzer, H. (1976) *Trends Biochem. Sci.* **1**, 178–181
 Jenkins, R. O., Cartledge, T. G. & Lloyd, D. (1983) *J. Gen. Microbiol.* **129**, 1171–1185
 Jenkins, R. O., Cartledge, T. G. & Lloyd, D. (1985) *J. Gen. Microbiol.* **131**, 335–344
 Lenney, J. F., Matile, P., Wiemken, A., Schellenberg, M. & Meyer, J. (1974) *Biochem. Biophys. Res. Commun.* **60**, 1378–1383
 Lenz, A. G. & Holzer, H. (1984) *Arch. Microbiol.* **137**, 104–108
 Lowry, O. H., Rosebough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
 Luck, H. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), vol. 2, pp. 885–900, Academic Press, New York
 Magni, G., Pallotta, G., Natalini, P., Ruggiero, S., Santarelli, I. & Vita, A. (1978) *J. Biol. Chem.* **253**, 2501–2503
 Matern, H., Hoffman, M. & Holzer, H. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4874–4878
 Mazon, M. J. (1978) *J. Bacteriol.* **133**, 780–785
 Roggkamp, R., Sahm, H., Hinkelmann, W. & Wagner, F. (1975) *Eur. J. Biochem.* **59**, 231–236
 Saheki, T. & Holzer, H. (1975) *Biochim. Biophys. Acta* **384**, 203–214
 Smith, L. (1955) *Methods Enzymol.* **2**, 736–740
 Switzer, R. L. (1977) *Annu. Rev. Microbiol.* **31**, 135–157
 Torriani, A. (1960) *Biochim. Biophys. Acta* **38**, 460–469
 van Dijken, J. P., Otto, R. & Harder, W. (1976) *Arch. Microbiol.* **111**, 137–144
 Veenhuis, M., Douma, A., Harder, W. & Osumi, M. (1983) *Arch. Microbiol.* **134**, 193–203
 Wolf, D. H. (1980) *Adv. Microb. Physiol.* **21**, 267–338
 Wolf, D. H. (1982) *Trends Biochem. Sci.* **7**, 35–37

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