Biochem. J. (1985) 232, 743–750 (Printed in Great Britain)

Degradative inactivation of the peroxisomal enzyme, alcohol oxidase, during adaptation of methanol-grown *Candida boidinii* to ethanol

David J. HILL,* Ao. C. HANN and David LLOYD
Department of Microbiology, University College, Newport Road, Cardiff CF2 1TA, Wales, U.K.

1. Adaptation of methanol-grown *C. boidinii* to ethanol-utilization in non-growing cells resulted in decreased activity of the peroxisomal enzyme alcohol oxidase. 2. Re-appearance of alcohol oxidase activity was dependent on protein synthesis *de novo*. 3. Degradation of alcohol oxidase protein was shown to parallel the decrease in activity. 4. Adaptation of methanol-grown cells to ethanol-utilization resulted in increased absorbance due to cytochromes and decreased absorbance due to flavoprotein. 5. Decrease in alcohol oxidase activity was associated with loss of the flavin coenzyme, FAD, from the organisms and the appearance of flavins (FAD, FMN, riboflavin) in the surrounding medium. 6. Electron microscopic observations showed that general degradation of whole peroxisomes rather than specific loss of crystalline cores (alcohol oxidase protein) occurred during the adaptation.

INTRODUCTION

Control of metabolism involves, not only regulation of enzyme synthesis and allosteric modulation, but also 'selective inactivation', defined as irreversible loss of enzyme activity *in vivo* in response to environmental change (Switzer, 1977). Two types of selective inactivation have been observed, (*a*) modification inactivation, in which the enzyme protein remains intact but loses its biological activity, and (*b*) degradative inactivation, in which at least one peptide bond of the protein is cleaved as part of the degradative process. Selective inactivation has been most extensively studied in *Saccharomyces cerevisiae* for glucose-induced inactivation of enzymes of the gluconeogenic pathway, namely cytoplasmic malate dehydrogenase, fructose 1,6-bisphosphatase and phosphoenolpyruvate carboxykinase, which are not required at high activities when glucose is presented as carbon source. The term 'catabolite inactivation' was proposed by Holzer (1976) to describe these processes. Although selective inactivation has been described for a variety of enzymes, the process is still poorly understood.

Selective inactivation of enzymes has recently been observed in methylotrophic yeasts when the carbon-source is changed from methanol to glucose (Veenhuis *et al.*, 1978). Growth of the yeast *C. boidinii* on methanol is associated with the development of large numbers of peroxisomes which contain high concentrations of catalase and the H_2O_2-producing flavoprotein, alcohol oxidase (Roggenkamp *et al.*, 1975). The latter enzyme is present as a crystal structure within the peroxisomes (Veenhuis *et al.*, 1981). In contrast, for growth in media containing either glucose or ethanol as carbon-source, proliferation of peroxisomes is not observed. Adaptation of methanol-grown *Hansenula polymorpha* to glucose metabolism has been associated with a concomittant loss of peroxisomes and of the activities of the peroxisomal enzymes alcohol oxidase and catalase (Veenhuis *et al.*, 1983).

In the present investigation we studied the adaptation of methanol-grown *C. boidinii* to ethanol-utilization, and showed that decrease in alcohol oxidase activity was associated with loss of the coenzyme FAD, which appeared in the extracellular medium mainly as FMN. The progress of degradative inactivation of alcohol oxidase was monitored by the disappearance of enzyme protein during peroxisomal degradation.

MATERIALS AND METHODS

Micro-organisms, culture conditions and adaptation to ethanol utilization

C. boidinii CBS5777 was grown at 30 °C with shaking in 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 reincubated with shaking at 30 °C. Cell growth was measured by absorbance increase at 663 nm.

Harvesting and breakage of the organism

Cells were harvested at 4 °C, unless stated otherwise, in a 6×250 ml rotor of an MSE 18 centrifuge at 8×10^4 *g*·min. Pellets were washed twice by resuspension in 50 mM-potassium phosphate buffer (pH 7.2).

Breakage was achieved using a Braun disintegrator (F.T. Scientific, Tewksbury, Glos., U.K.) operating at 4000 Hz for 4 min with liquid CO_2 cooling. Whole cells were removed by centrifugation at 8×10^4 *g*·min and the supernatant was used without further fractionation.

Enzyme assays

Alcohol oxidase (EC 1.1.3.13) activity in whole cells and extracts was determined polarographically according to van Dijken *et al.* (1976).

Abbreviation used: SDS, sodium dodecyl sulphate.

* Present address: Biotechnology Unit, University of Leeds, Leeds LS2 9JT U.K.

Difference spectra

Difference spectra were obtained from suspensions of intact organisms under a gas phase of precisely designed composition in a scanning dual-wavelength spectrophotometer (Hitachi–Perkin-Elmer, model 557). A cuvette, moulded from epoxy resin, was hexagonal in cross-section, with quartz windows and 16 mm path length. Suspensions (7 ml) were maintained at 30 °C, and stirring was by a cross-shaped stirrer fixed to a stainless steel shaft entering through a hole in the lid and driven by a synchronous motor at 750 rev./min. The membrane-covered oxygen electrode (Radiometer, Copenhagen, Denmark) was sealed into a port lying below the level of the vortex (Degn *et al.*, 1980). The half time for equilibration with O_2 was slightly less than 3 min corresponding to an O_2-transfer coefficient of 0.23 min^{-1}.

Spectrophotofluorimetry

Flavin fluorescence was measured (454→543 nm) using a fluorescent spectrophotometer (Applied Photophysics, London, U.K.), connected to an *x–y* chart recorder. At fixed wavelengths and in scanning mode, excitation and emission monochromator slit widths were both 2 mm, photomultiplier voltage was 1.75 kV.

Thin layer chromatography

T.l.c. of flavins was carried out according to Fazekas & Kokai (1971). Samples were applied to silica gel plates (Merck) and developed in an Eastman chromatogram-developing apparatus (model 104, Kodak, London, U.K.). After developing, plates were dried and the spots were observed under a u.v. light source.

Immunological determination of alcohol oxidase

Anti-(alcohol oxidase) serum was prepared by injection of 25 units (2.5 mg of protein) of purified alcohol oxidase (EC 1.1.3.13), obtained from Sigma, in Freund's complete adjuvant into one rabbit as follows: intramuscularly, subcutaneously, and intraperitoneally. The injection was repeated 18 days later and a further 14 days later the rabbit was bled endocardially. The serum obtained, after removal of red blood cells by centrifugation, was stored at −20 °C until required.

Alcohol oxidase (EC 1.1.3.13) was determined by titration against the serum using the complement-fixation technique.

Polyacrylamide-gel electrophoresis

Samples were run on vertical gels 0.75 mm thick with a resolving: stacking gel ratio of 2:1. For non-dissociating electrophoresis, the stacking gel was 3.75% polyacrylamide (pH 6.8), the resolving gel was 7.5% polyacrylamide (pH 8.8), and the reservoir buffer was Tris/HCl (pH 8.3). For electrophoresis in SDS, the stacking gel was 3.75% polyacrylamide (pH 6.8) containing 0.1% SDS, the resolving gel was 10% polyacrylamide (pH 8.8) containing 0.1% SDS, and the reservoir buffer was Tris/glycine (pH 8.3) containing 0.1% SDS. Gels were run at 20 °C at an operating voltage of 120 V (Laemmli, 1970). Gels were fixed and stained for protein by incubation with 0.1% (w/v) Coomassie Blue in water/methanol/glacial acetic acid (5:5:2, by vol.) for 30 min. After destaining, gel bands were scanned using an LKB 2202 Ultrascan laser densitometer and peak areas of bands were measured.

Electron microscopy

Samples for electron microscopy in 0.05 M-potassium phosphate buffer (pH 7.0) were fixed with equal volumes of 3% (v/v) glutaraldehyde for 1–2 h at 4 °C. Post-fixation was with 1% (w/v) OsO_4 in the same buffer for 1 h at 4 °C. Following dehydration, the samples were embedded in Spurr's resin and sections were cut with a Reichart ultramicrotome. Sections were stained with 2% uranyl acetate in 50% (v/v) ethanol for 30 min at 20 °C in the dark, and counterstained with lead citrate for 5 min. Examination of sections was in a Philips electron microscope operating at 80 kV.

Other assays

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

The bacterial luciferase assay for FMN and preparation of bacterial luciferase was according to Chapelle & Picciolo (1971). The reaction vessel contained 200 μl of luciferase solution (saturated with dodecylaldehyde) and 50 μl of the sample to be assayed. Samples, reduced with $NaBH_4$, were injected into the system through a light-sealed stopper. Light emission was measured in a luminometer connected to a chart recorder.

RESULTS

Changes in alcohol oxidase activity and protein levels during adaptation of methanol-grown *C. boidinii* to ethanol

Fig. 1(*a*) shows the decline in alcohol oxidase activity in cell free extracts prepared at intervals during the adaptation of a washed cell suspension of methanol grown *C. boidinii* to ethanol utilization. Over a period of 9 h the activity declined exponentially and became undetectable: the $t_{\frac{1}{2}}$ for loss of enzyme activity was 1.45 h. Younger cultures are inactivated at higher rates (Veenhuis *et al.*, 1984). That this process involved inactivation of enzyme was confirmed by inhibition by cycloheximide of restoration of methanol oxidation capacity when organisms adapted to ethanol for 6 h were resuspended in methanol-containing growth medium.

To elucidate the methanism of inactivation, an immunossay system was used to determine alcohol oxidase protein using antiserum prepared against commercially obtained (Sigma) *C. boidinii* alcohol oxidase. Single precipitation lines were obtained both with the commercial alcohol oxidase and with a cell-free extract prepared from methanol-grown *C. boidinii* at varying antiserum:antigen ratios. Cell-free extracts prepared from samples of *C. boidinii* undergoing adaptation to ethanol were immunologically titrated for alcohol oxidase protein. Immunologically detectable alcohol oxidase protein initially declined more slowly ($t_{\frac{1}{2}}$ = 2.8 h) and lagged significantly behind the decrease in enzyme activity. These results provide evidence for the degradative inactivation of alcohol oxidase during adaptation to ethanol-utilization, possibly with the formation of an inactive intermediate prior to enzyme degradation. These changes occurred in the absence of net cell growth as determined by measurement of A_{663}.

Incubation of methanol-grown cells in potassium phosphate buffer (pH 6.0) also gave a decrease in activity, but only 40% of the activity was lost after 7.5 h (Fig. 1*b*). Immunological determination of alcohol oxidase protein

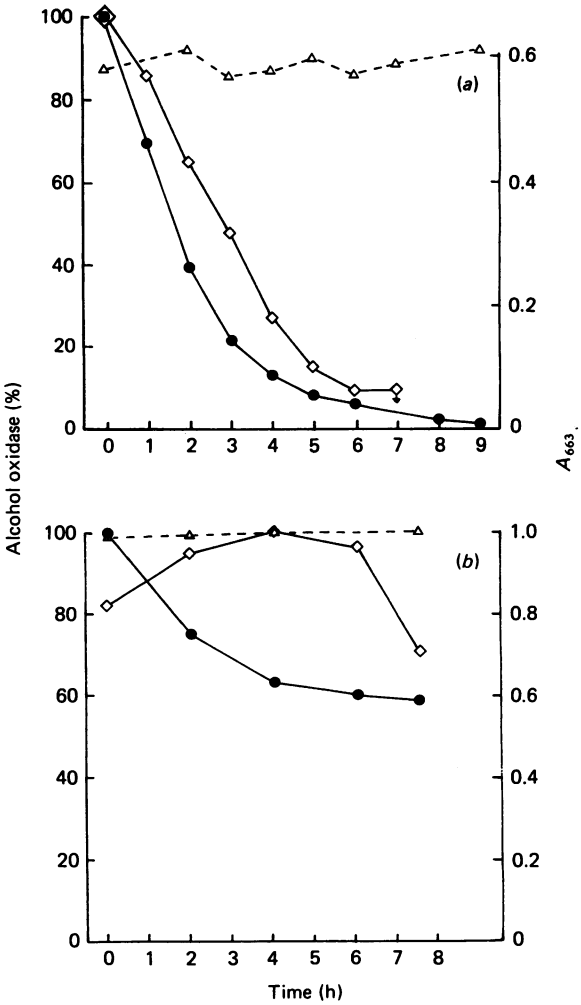

Fig. 1. Loss of alcohol oxidase protein during adaptation of methanol-grown *C. boidinii* to ethanol-utilization

(*a*) Methanol-grown cells were washed and aerated at 30 °C in potassium phosphate buffer (pH 6.0) containing 174 mM-ethanol. Alcohol oxidase activity (●) of cell-free extracts was determined by measurement of O_2 uptake in the presence of 33 mM-methanol. Alcohol oxidase protein (◇) in cell-free extracts was estimated by immunological titration against rabbit anti-(alcohol oxidase) serum using complement-fixation. Absorbance of the cell suspension at 663 nm (△) is also shown. (*b*) As in (*a*) but cells incubated in the absence of ethanol.

indicated that the decrease in activity in the absence of ethanol over the first 6 h was not due to enzyme degradation.

Since dissociation to subunits or other alterations in a protein may render it immunologically undetectable, an independent assay for the disappearance of alcohol oxidase protein was carried out. Polyacrylamide-gel electrophoresis was employed. On non-dissociating electrophoresis of purified alcohol oxidase (obtained from Sigma), it ran as a single band with an R_m value of 0.10 which matched the major band appearing on electrophoresis of cell-free extracts of methanol-grown *C. boidinii*. Further identification of this band as alcohol oxidase was achieved by enzyme staining in the gel using diaminobenzamidine. The R_m values determined for

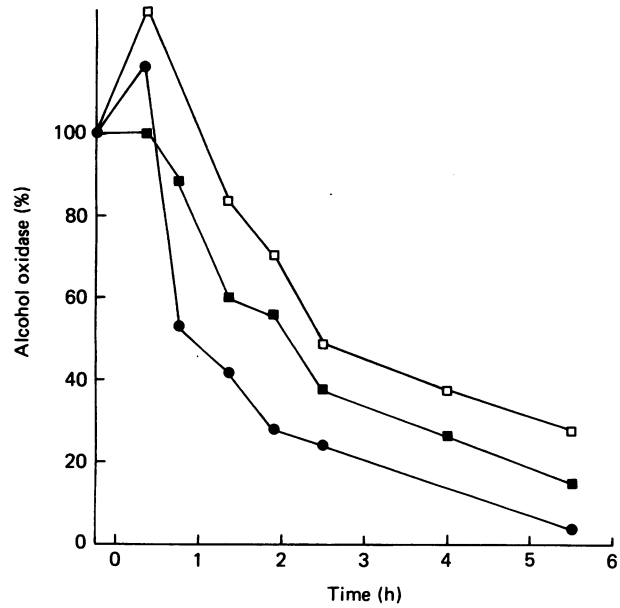

Fig. 2. Alcohol oxidase protein during adaptation of methanol-grown *C. boidinii* to ethanol-utilization, as determined by gel electrophoresis

Alcohol oxidase activity (●) was determined by oxygen uptake in the presence of 33 mM-methanol for cell-free extracts. Alcohol oxidase protein was determined on non-dissociating (□) and SDS (■) polyacrylamide-gel electrophoresis from peak areas of Coomassie Blue-stained gels after laser densitometry at 550 nm.

alcohol oxidase in 7.5% polyacrylamide gels were identical with the value determined in a previous work describing polyacrylamide-gel electrophoresis of alcohol oxidase from *C. boidinii* (Lee & Komagata, 1983).

Similarly, SDS/polyacrylamide-gel electrophoresis of purified alcohol oxidase (Sigma) gave a single band with an R_m value of 0.38 which matched the major protein band after electrophoresis of cell-free extracts of methanol-grown cells. Absorbance measurements on scanned gels were linear up to 0.4 units (40 μg of protein) of the enzyme for both non-dissociating and SDS electrophoresis. Fig. 2 shows that decreases in alcohol oxidase protein as determined by non-dissociating and SDS electrophoresis ($t_{\frac{1}{2}} = 1.6$ h) followed the decrease in alcohol oxidase activity with a lag between the decrease in activity and the decrease in enzyme protein. These results confirm those obtained by the immunological determination of alcohol oxidase protein (Fig. 1).

Electron microscopical observation

Samples of methanol-grown cells undergoing adaptation to ethanol were prepared for electron-microscopy according to the method of Bormann & Sahm (1978). Fig. 3 shows events occurring during the adaptation over a 6 h period. A typical methanol-grown cell (Fig. 3*a*) contained many large peroxisomes each with a definite membrane and a well-defined lattice structure. The first stage of adaptation to ethanol utilization involved a decrease in the definition of peroxisomal structure (Fig. 3*b*), especially that of the crystal lattice which appeared to be merging into the rest of the peroxisome. Clear definition of individual peroxisomes became difficult to observe.

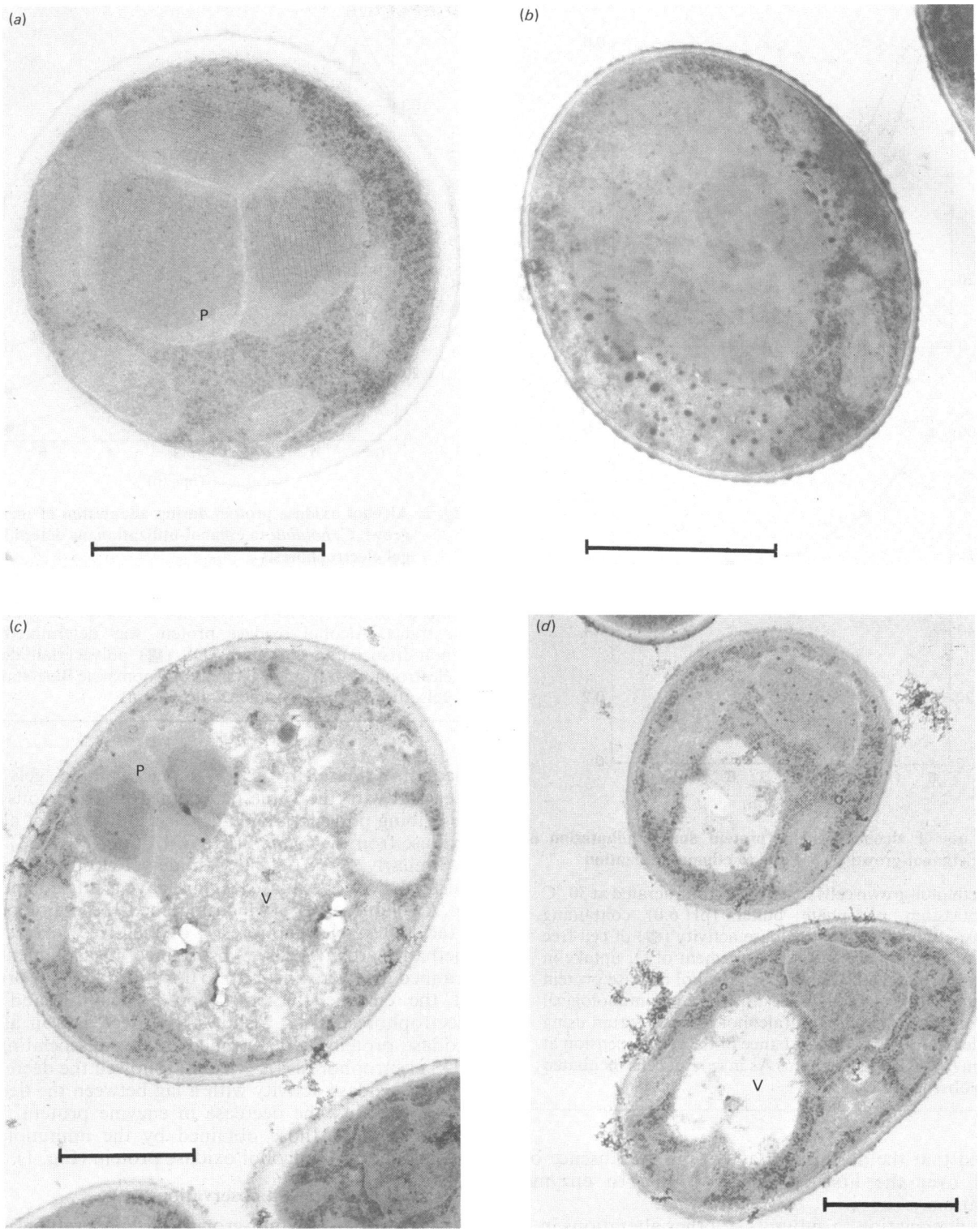

Fig. 3. Electron micrographs of sections of *C. boidinii* grown on methanol and during adaptation to ethanol-utilization

(*a*) Electron micrographs of methanol-grown *C. boidinii* and of methanol-grown cells during adaptation to ethanol for 2 h (*b*), 4 h (*c*) and 6 h (*d*), showing peroxisomes (P) and vacuole (V). The marker represents 1 μm.

These changes were followed by the appearance of large vacuoles (Fig. 3*c*), which were often associated with the remaining peroxisomes. The typical appearance of many cells after 6 h adaptation to ethanol (Fig. 3*d*) showed large vacuolar areas without peroxisomes. Mean peroxisome numbers per cell profile decreased from 3.3 to 1.5 over a period of 6 h (100 cell profiles examined before and after adaptation). Changes in ultrastructure of other cellular organelles (e.g. nuclei or mitochondria) were not observed during adaptation to ethanol.

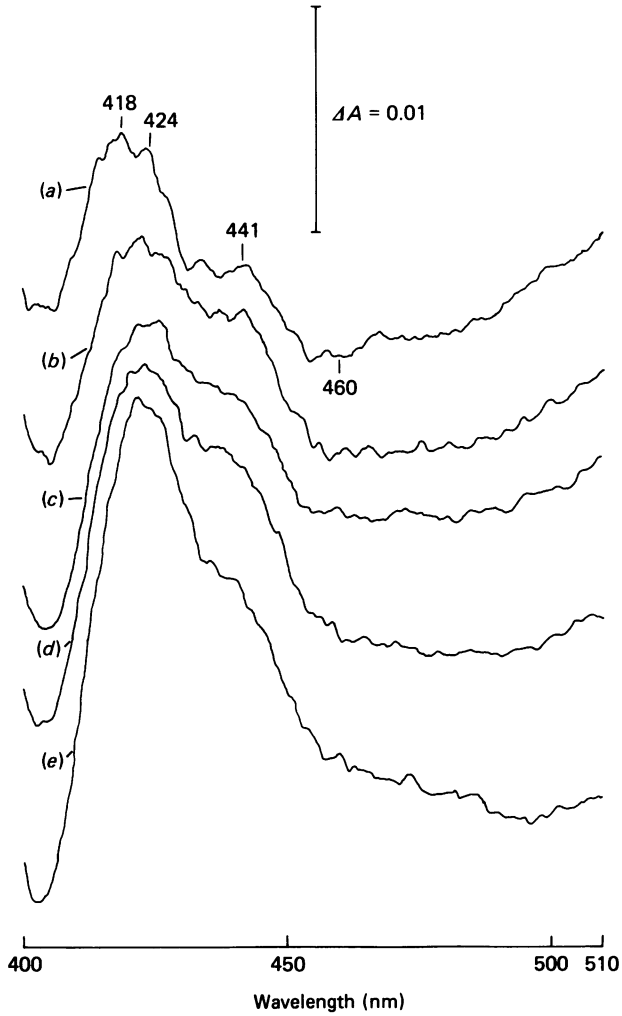

Fig. 4. Steady-state difference spectra of methanol-grown *C. boidinii* during adaptation to ethanol-utilization

Difference spectra of washed cells (dithionite reduced–aerated) were recorded at (*a*) 0 min, (*b*) 20 min, (*c*) 73 min, (*d*) 190 min and (*e*) 540 min after transfer of methanol-grown cells into ethanol-containing buffer. The temperature was 30 °C, the path-length 1.6 cm, the reference wavelength 510 nm, the spectral band-width 1.5 nm, the scan speed 120 nm/min and the O_2 concentration under aerobic conditions was > 10 μM.

Spectral changes of cytochromes and flavins during adaptation of methanol-grown cells to ethanol utilization

Fig. 4 shows the difference spectra (dithionite reduced-aerated) recorded at 30 °C of samples taken at 0, 20, 72 and 540 min of adaptation of methanol-grown cells to ethanol showing decreasing absorbance minima at 460 nm due to flavoprotein and increasing absorbance maxima at 428 and 441 nm for cytochromes *b* and aa_3 respectively. Correlation of these spectral changes with methanol-supported respiration of whole cells and the alcohol oxidase activities of cell-free extracts is shown in Fig. 5. The decreases in the respiratory capacity for methanol and alcohol oxidase activity were paralleled by a similar decrease in absorbance due to flavoprotein and therefore reflects changes in the flavoprotein alcohol oxidase. Increase in absorbance due to cytochromes during adaptation indicates mitochondrial development during the process. Ethanol-grown cells show approximately twice the mitochondrial cytochrome content of methanol-grown organisms. Fig. 5 also shows release of flavin from organisms into the surrounding medium during the adaptation of methanol-grown cells to ethanol, as determined from fluorescence emission spectra.

Intracellular flavoprotein content, as determined from the absorbance change of the minima (460–510 nm), using a value of 11 $mM^{-1} \cdot cm^{-1}$ for the absorption coefficient (reduced – oxidized) (Chance, 1957), gave a calculated net change in flavin content of 61 nM for the cell suspension during adaptation. Comparison of this decrease in intracellular flavin with the increase in extracellular flavin (as determined by fluorescence spectroscopy) revealed that the decrease in intracellular flavin can be totally accounted for by its excretion into the surrounding medium. Bruinenberg *et al.* (1982) have previously shown that glucose-inactivation of alcohol oxidase in *H. polymorpha* is accompanied by extracellular flavin accumulation.

Analysis of extracellular flavin

Photolysis of flavin liberated during adaptation was prevented by performing the adaptation and the subsequent extraction in the dark. Hydrolysis of FAD was shown not to occur during the procedures.

The pH dependence of fluorescence emission was determined for FMN, FAD and the extracellular flavin after concentration by phenol extraction. Fig. 6

Table 1. Chromatographic properties of flavin released from methanol-grown *C. boidinii* during adaptation to ethanol

Extracellular flavins were concentrated 100-fold by phenol extraction and chromatographed on silica gel in the following solvents: I, butan-1-ol/water/glacial acetic acid/methanol (7:7:0.5:3, by vol.); II, butan-2-ol/glacial acetic acid/water (12:3:5, by vol.); III, 5% (w/v) Na_2HPO_4, 12 H_2O in water; IV, the lower phase of 160 g of phenol/30 ml of butan-1-ol/100 ml of water.

	100 × R_F values in solvent system:			
Sample	I	II	III	IV
Extracellular flavin	64, 78.4	12.5, 25, 38.5, 51	35.1, 51.5, 60, 68.2	5.3, 10.7, 53.3
FAD	62.7	12.5	68.2	12
FMN	65.4	25.2	51.5	12.7
Riboflavin	78.4	50.7	36.6	54.7

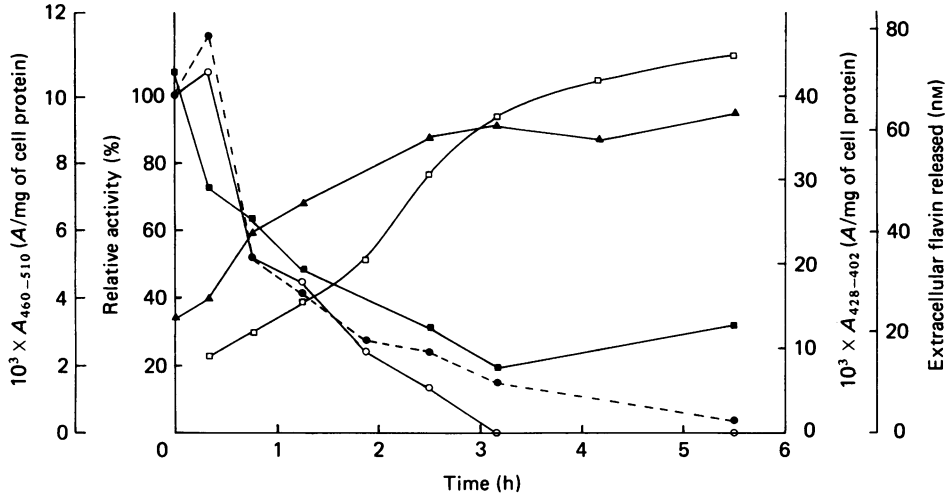

Fig. 5. Changes in alcohol oxidase activity, cellular and cytochrome and flavin contents and extracellular flavin during adaptation of methanol-grown *C. boidinii* to ethanol

Methanol-grown cells were washed and aerated at 30 °C in potassium phosphate buffer (pH 6.0) containing 174 mM-ethanol. Alcohol oxidase activity was polarographically determined for whole cells (○), and extracts (●). Absorbance due to *b*-type cytochromes (▲) and flavoprotein (■) were determined from steady-state difference spectra (Fig. 4). Extracellular flavin released (□) was determined fluorimetrically (454→531 nm).

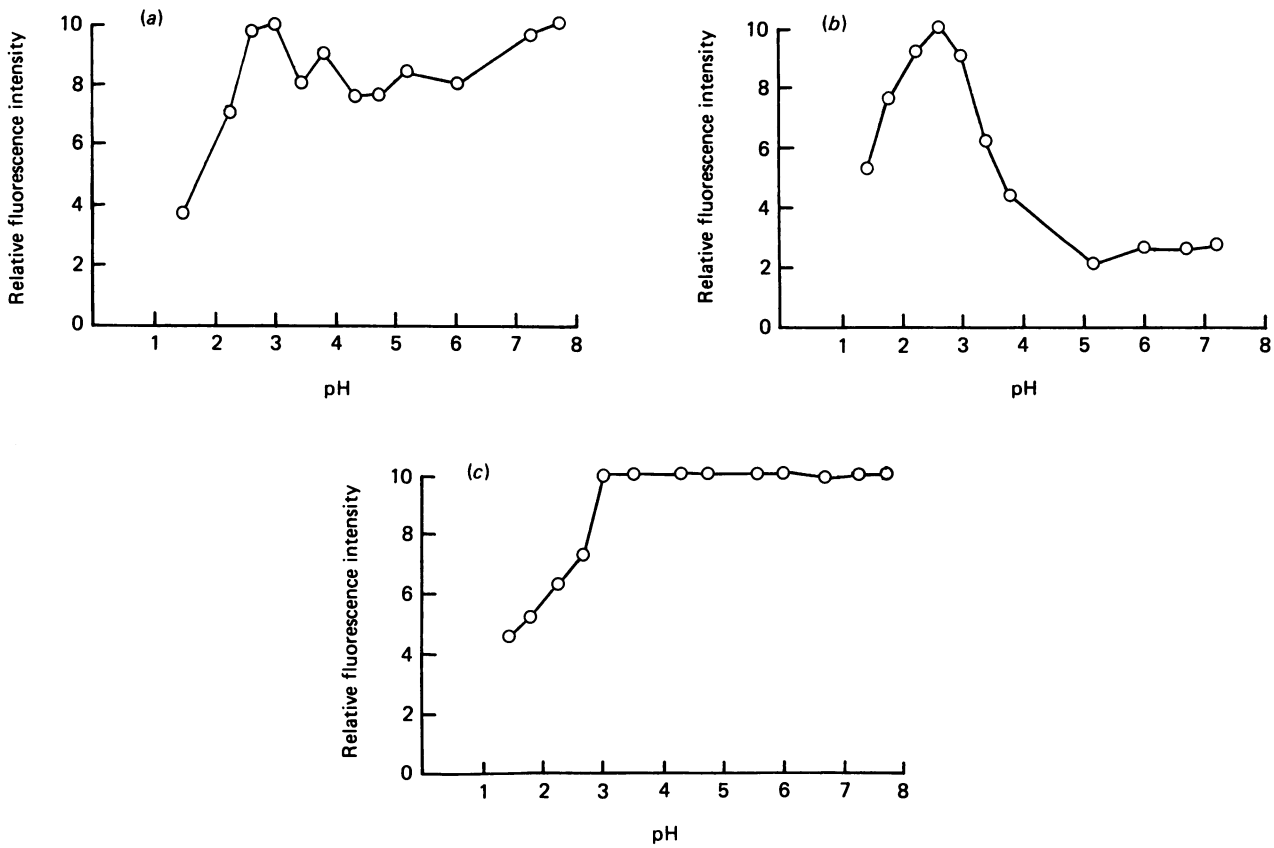

Fig. 6. pH-dependence of fluorescence of flavin released from *C. boidinii* during ethanol adaptation

(*a*) Extracellular flavin released during the adaptation of methanol-grown cells to ethanol was concentrated by the phenol extraction procedure and fluorescence emission (454→531) was determined: buffers used were HCl/KCl buffer (pH 1.0–2.2); glycine buffer (pH 2.2–3.6); acetate buffer (pH 3.6–5.6); citrate buffer (pH 3.0–7.0) and phosphate buffer (pH 5.7–8.0), all 100 mM. (*b*) Flavin adenine dinucleotide (100 nM). (*c*) Riboflavin 5-phosphate (100 nM).

shows that whereas the fluorescence of FMN (454 nm→531 nm) increases from pH 1.0 to about pH 3.0 and remains constant at pH values > 3.0, FAD shows increased fluorescence from pH 1.0 to pH 2.6 and a decrease to about 20% of the maximal fluorescence between pH 2.6 and pH 5.0. The extracellular flavin also shows increase in fluorescence up to pH 2.6–3.0, decreased to 80%, and then gradually increased to its maximal fluorescence at pH 8.0. These results suggest that the extracellular flavin was neither totally FMN nor FAD.

T.l.c. of standard solutions of riboflavin, FMN and FAD was carried out alongside the unknown flavin. R_F values (Table 1) suggest that the excreted flavin was predominantly riboflavin and FMN, with very little FAD present, and a trace of a fourth unidentified flavin.

To confirm that the extracellular flavin sample contained FMN, the bacterial luciferase assay was set up, in which bioluminescence produced by the luciferin-luciferase reaction is dependent on reduced FMN. Comparison with FMN standards and total fluorescence measurements indicated that 55% of the extracellular flavin was FMN.

DISCUSSION

The present investigation has shown that adaptation of methanol-grown *C boidinii* to ethanol utilization results in an irreversible decrease in the activity of the peroxisomal enzyme alcohol oxidase, and this inactivation of alcohol activity was associated with a loss in immunologically detectable enzyme protein. In the absence of ethanol the smaller decrease in activity observed was not associated with a decrease in immunologically determined protein, indicating that ethanol metabolism is in some way responsible.

The processes leading to decreased activity and enzyme protein were such that the observed decline in activity preceded the decrease in alcohol oxidase protein, probably as a consequence of a difference in initial rates. This suggests that an initial enzyme modification inactivation may be involved prior to its degradation. During glucose inactivation of fructose-1,6-bisphosphatase and NADP-dependent glutamate dehydrogenase an initial reversible phosphorylation occurs prior to degradation (Mazon *et al.*, 1982; Hemmings, 1982). On the other hand, ornithine aminotransferase is substantially more susceptible to degradation upon removal of the pyridoxal phosphate coenzyme to yield the apoenzyme (Katanuma *et al.*, 1978). Modifications of these types result in changes in protein configuration which expose certain amino acid sequences for cleavage.

A third possible type of modification involved in loss of alcohol oxidase activity may have resulted from dissociation of the protein into its subunits prior to degradation. However, comparison of the levels of alcohol oxidase protein determined by non-dissociating polyacrylamide-gel electrophoresis with the total subunit levels as determined by electrophoresis in the presence of SDS did not reveal any evidence to support this hypothesis.

To elucidate the mechanism of inactivation the spectral changes occurring during the adaptation were followed by difference spectrophotometry of whole cells. During adaptation of methanol-grown cells to ethanol-utilization, mitochondrial development occurred as shown by increase in *c*- and *a*-type cytochrome absorbance. Decrease in methanol respiration and alcohol oxidase activity was associated with decreased absorbance due to flavoprotein and was attributed to loss of the flavin coenzyme from alcohol oxidase into the surrounding medium. Analysis of the excreted flavin revealed it to be a mixture of flavins; FMN was predominant but some riboflavin was also detected. Thus hydrolysis of released FAD occurred, and presumably phosphatases (including phosphomonesterase and phosphodiesterase) were responsible. Whether this hydrolysis of FAD is intra- or extracellular is not known.

The degradation of alcohol oxidase protein during the adaptation to ethanol requires proteinase action. Electron microscopical studies suggested a general peroxisomal degradation effected by a vacuolar process, rather than an induction within peroxisomes of a proteolytic system specific for degradation of the enzyme. A vacuolar process has been proposed for peroxisomal degradation in *H. polymorpha* during glucose-inactivation of methanol-grown cells, based on electron-microscopical studies and cytochemical staining (Veenhuis *et al.*, 1983). Vacuolar involvement seems likely, since in yeasts the vacuole is known as the principal cellular compartment of proteolytic enzymes (Wiemken *et al.*, 1979; Wolf, 1980).

Further work is required to characterize the control of events responsible for initiating FAD detachment and the mechanism of proteolytic activation.

REFERENCES

Bormann, C. & Sahm, H. (1978) Arch. Microbiol. **117**, 67–72

Bruinenberg, P. G., Veenhuis, M., van Dijken, J. P., Duine, J. A. & Harder, W. (1982) FEMS Microbiol. Lett. **15**, 45–50

Chance, B. (1957) Methods Enzymol. **4**, 273–329

Chapelle, E. W. & Picciolo. G. L. (1971) Methods Enzymol. **18**, 381–384

Degn, H., Lundsgaard, J. S., Petersen, L. C. & Ormicki, A. (1980) Methods Biochem. Anal. **26**, 47–77

Fazekas, A. G. & Kokai, K. (1971) Methods Enzymol. **18**, 385–398

Haywood, G. W. & Large, P. J. (1981) Biochem. J. **199**, 187–201

Hemmings, B. A. (1982) Biochem. Soc. Trans. **10**, 328–329

Holzer, H. (1976) Trends Biochem. Sci. **1**, 178–181

Katanuma, N., Kominami, E. & Sanada, Y. (1978) in Limited Proteolysis in Microorganisms (Cohen, G. N. & Holzer, H., eds.), pp. 27–32, U.S. DHEW, Washington DC

Laemmli, U. K. (1970) Nature (London) **227**, 680–685

Lee, J. D. & Komagata, K. (1983) J. Gen. Appl. Microbiol. **29**, 395–416

Lowry, O. H., Rosebough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275

Mazon, M. J., Gancedo, J. M. & Gancedo, C. (1982) J. Biol. Chem. **257**, 1128–1130

Roggenkamp, R., Sahm, H., Hinkelmann, W. & Wagner, F. (1975) Eur. J. Biochem. **59**, 231–236

Switzer, R. L. (1977) Annu. Rev. Microbiol. **31**, 135–157

van Dijken, J. P., Oostra-Demkes, G. J., Otto, R. & Harder, W. (1976) Arch. Microbiol. **111**, 137–144

Veenhuis, M., van Dijken, J. P., Pilon, S. A. F. & Harder, W. (1978) Arch. Microbiol. **117**, 153–163

Veenhuis, M., Zwart, K. B. & Harder, W. (1981) Arch. Microbiol. **129**, 35–41

Veenhuis, M., Douma, A., Harder, W. & Osumi, M. (1983) Arch. Microbiol. **134**, 193–203
Veenhuis, M., van Dijken, J. P. & Harder, W. (1984) Adv. Microb. Physiol. **24**, 7–82
Wiemken, A., Schellenberg, M. & Urech, K. (1979) Arch. Microbiol. **123**, 23–35
Wolf, D. H. (1980) Adv. Microb. Physiol. **21**, 267–338

Received 3 June 1985/31 July 1985; accepted 19 August 1985