Import of alcohol oxidase into peroxisomes of *Saccharomyces cerevisiae*

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Saccharomyces cerevisiae is unable to grow on methanol because it lacks the enzymes required for its metabolism. To study the possibility of whether or not the methanol oxidation pathway of *Hansenula polymorpha* can be transferred to *S. cerevisiae*, the gene coding for alcohol oxidase, a peroxisomal homo-octameric flavoprotein, was introduced into *S. cerevisiae*. Transformed cells contain varying amounts of alcohol oxidase depending on the plasmid used. Immunocytochemical experiments indicate that the protein is imported into peroxisomes, whether organelle proliferation is induced or not. Cells lack alcohol oxidase activity however, and only the monomeric, non-functional, form of the protein is found. These findings indicate that the *H. polymorpha* peroxisomal targeting signal of alcohol oxidase is recognized in *S. cerevisiae* and protein monomers are imported.
Key words: alcohol oxidase/peroxisome/protein targeting/ *Saccharomyces cerevisiae*

Introduction

In eukaryotes proteins are synthesized on membrane-bound or free ribosomes and subsequently directed to their subcellular destination. Two modes of protein routing can be distinguished. One is mainly co-translational, delivering proteins into the endoplasmic reticulum and Golgi apparatus from which vesicles can pinch off, which either discharge their contents into the extracellular space or contribute to organelle assembly. The other is posttranslational, in which finished translation products enter the subcellular compartment directly, e.g. mitochondria, nuclei or chloroplasts. In both pathways specific amino acid sequences, often located at the N-terminus of the protein, are involved in targeting of proteins to subcellular compartments, and translocation across the membrane of the target organelle is usually coupled to cleavage of the topogenic signal. Relatively little, however, is known with respect to topogenic signals of peroxisomal proteins. Moreover, the absence of signal peptide removal upon arrival in the organelle offers no simple clues as to where the topogenic signal is located within peroxisomal proteins (Lazarow and Fujiki, 1985; Borst, 1986).

We have studied the routing of a heterologous peroxisomal protein in *Saccharomyces cerevisiae* to investigate whether it can be addressed to the intended location within the cell and attain its functional form. In this way it might be possible to add a new enzyme activity or even a new metabolic pathway to the existing metabolic repertoire of this organism. The gene we selected for introduction into *S. cerevisiae* codes for alcohol oxidase, an enzyme involved in methanol metabolism in *Hansenula polymorpha*. Microorganisms capable of utilizing methanol as the

sole source of energy occur abundantly in nature; however, only a few methanol-utilizing yeast species have been identified (for review see Veenhuis *et al.*, 1983). In all methylotrophic yeast strains studied thus far (e.g. *H. polymorpha*) the conversion of methanol to formaldehyde (and H_2O_2) is catalyzed by an alcohol oxidase, a peroxisomal flavoprotein which consists of eight identical subunits and has a mol. wt of ~640 kd (Sahm, 1977; Veenhuis *et al.*, 1976). Depending on the growth conditions the enzyme may be present in very high amounts, up to 30% of the total soluble protein (Veenhuis *et al.*, 1983). Here we show that the *H. polymorpha* gene coding for alcohol oxidase, provided with a *S. cerevisiae* promoter, can be expressed in *S. cerevisiae* and that the protein is imported into peroxisomes of this yeast. Since alcohol oxidase is a non-essential protein in *S. cerevisiae* which can be altered at liberty, it is an excellent tool to explore the nature of the still unknown peroxisomal import signal.
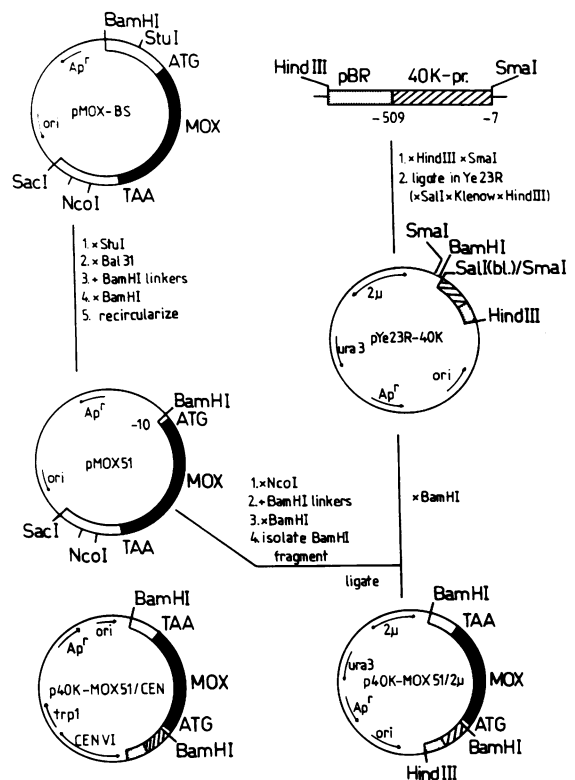

Fig. 1. Construction of the p40K-MOX51 expression plasmids. The plasmid pMOX-BS, containing the entire alcohol oxidase structural gene, was digested with *Bal*31 to remove most of the 5' untranslated sequences. One of the deletion clones (pMOX51, extending to position −10) was fused with a fragment containing a *S. cerevisiae* promoter (of the 40 kd subunit of complex III) and cloned in a multi-copy shuttle plasmid Ye23R (p40K-MOX51/2μ) and a single-copy plasmid pLA433 (p40K-MOX51/CEN) (for details see Materials and methods). Open boxes, 5' and 3' untranslated alcohol oxidase sequences; closed box, alcohol oxidase coding sequence; hatched box, 40K promoter fragment; dotted box, pBR322 sequences.
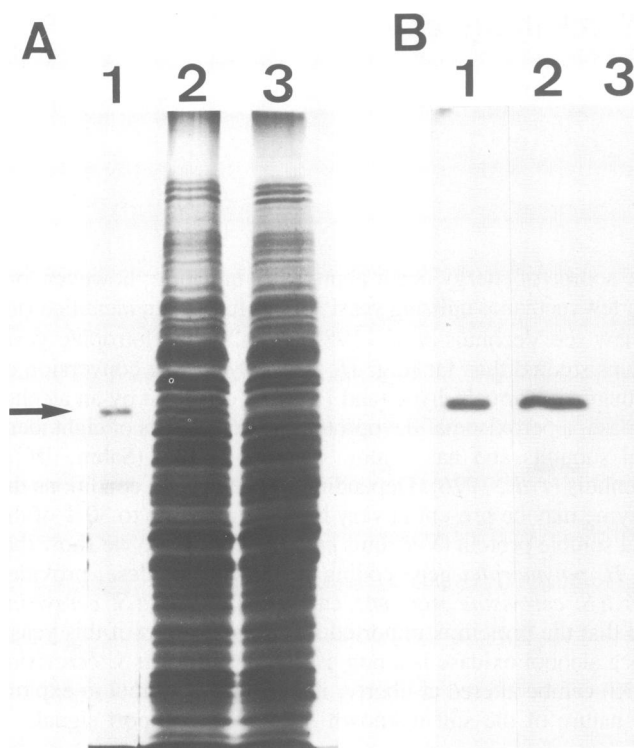
A

B

Fig. 2. Expression of the alcohol oxidase gene. Protein extracts of cells grown in minimal medium with ethanol as a carbon source (WOYE) were subjected to SDS—polyacrylamide gel electrophoresis and stained with Coomassie brilliant-blue (**A**) or blotted onto nitrocellulose and incubated with anti-alcohol oxidase antibody and ^{125}I-labeled goat-anti-rabbit antiserum (**B**). Lane 1, purified alcohol oxidase from *H. polymorpha*; lane 2, p40K-MOX51/2μ transformed cells; lane 3, Ye23R transformed cells.

Table I. Relative alcohol oxidase expression levels

Plasmid	Carbon source	
	Ethanol	Oleic acid
p40K-MOX51/2μ	6–7	5–6
p40K-MOX51/CEN	1	1

Yeast cells transformed with the multi-copy (p40K-MOX51/2μ) or single-copy (p40K-MOX51/CEN) recombinant plasmid were grown on minimal medium (WOY) with ethanol or oleic acid as carbon source. Total cellular protein was titrated on SDS—polyacrylamide gels and alcohol oxidase was detected by Western blotting. Relative alcohol oxidase expression levels were calculated by cutting out the bands and counting the radioactivity. Values are normalized for the expression found with the single-copy plasmid.

Results

Expression of the H. polymorpha gene coding for alcohol oxidase in S. cerevisiae

To minimize possible problems in the control of expression, the cloned *H. polymorpha* gene coding for alcohol oxidase was retailored and put behind a *S. cerevisiae* promoter belonging to the gene coding for the 40 kd subunit of the ubiquinol—cytochrome *c* reductase complex (complex III) (Oudshoorn *et al.*, 1987). The construct was introduced into yeast cells on a multi-copy plasmid (p40K-MOX51/2μ) or a single-copy centromere containing plasmid (p40K-MOX51/CEN) (Figure 1) and transformants were tested for alcohol oxidase expression.

Transformed yeast cells, grown on ethanol as a carbon source to obtain optimal expression from the 40K promoter, were lysed

and protein extracts were prepared for analysis by SDS—polyacrylamide gel electrophoresis. The results, shown in Figure 2A, reveal the presence of an extra polypeptide in extracts of p40K-MOX51/2μ transformed cells (lane 2) which comigrates with the 75 kd subunit of purified alcohol oxidase from *H. polymorpha* (lane 1). This protein is absent from extracts of cells transformed with the vector alone (lane 3). After transfer of separated proteins from cell-free extracts to nitrocellulose and incubation with antiserum raised against alcohol oxidase, a single protein is detected in transformed cells (Figure 2B, lane 2 versus 3) with the same molecular weight as found for purified alcohol oxidase (Figure 2B, lane 1). The level of expression was estimated to be 1−3% of total yeast protein. Essentially the same results were obtained with cells transformed with a single-copy plasmid (p40K-MOX51/CEN) although the levels of expression were a factor five to seven lower depending on growth conditions (Table I).

Subcellular location of alcohol oxidase protein

The subcellular morphology of p40K-MOX51 transformed and control cells was studied in ultrathin sections and the location of the alcohol oxidase protein was determined by immunocytochemistry. Apart from the usual cell organelles (nuclei, vacuoles, mitochondria and low numbers of peroxisomes), large electron-dense inclusions in the cytoplasm are visible in cells transformed with the multi-copy recombinant plasmid (p40K-MOX51/2μ) (Figure 3B). In control cells (Figure 3A) and in p40K-MOX51/CEN transformed cells (see below, Figure 5D) cytoplasmic inclusions are not detected. These inclusions developed at the end of logarithmic growth. Immunocytochemistry shows that these cytoplasmic inclusions contain alcohol oxidase protein (Figures 3D and 5C). This protein is also present in the small number of peroxisomes of ethanol-grown cells (Figure 3C).

Peroxisome proliferation and synthesis of the β-oxidation enzymes required for the catabolism of fatty acids can be induced in *S. cerevisiae* by transferring cells to oleic acid (Veenhuis *et al.*, 1987; Kunau *et al.*, 1987). *S. cerevisiae* transformed with the alcohol oxidase gene on the single copy plasmid (p40K-MOX51/CEN) was grown on glucose to early exponential phase and subsequently inoculated into a medium with 0.1% oleic acid as the sole source of carbon. At different time points after the shift samples were withdrawn for determination of alcohol oxidase synthesis and for inspection by electron microscopy. Cells of the inoculum contained very low levels of alcohol oxidase due to repression of the mitochondrial 40K promoter by glucose. After the shift however, the amount of alcohol oxidase showed a steady increase (Figure 4). A simultaneous induction of peroxisome proliferation was observed in the electron microscope leading to an increased number of peroxisomal profiles per cell with enlarged volumes compared to the organelles present in the inoculum cells (Figure 5A). The organelles were cytochemically characterized as being peroxisomes by the presence of catalase (Figure 5B). Immunocytochemistry revealed that alcohol oxidase protein is present within organelles bounded by a single membrane in numbers equivalent to the induced peroxisomes both in p40K-MOX51/CEN and p40K-MOX51/2μ transformed cells (Figure 5C and D). Immunocytochemistry with antibodies specific for alcohol oxidase and the bifunctional enzyme of the peroxisomal β-oxidation pathway (comprising enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase), each marked with different sized gold particles, demonstrates that the alcohol oxidase is indeed located within peroxisomes since both antibodies decorate the same organelles (see Figure 5D, insert). Similar results were obtained in a double labeling experiment using antibodies against catalase and alcohol oxidase (not shown).
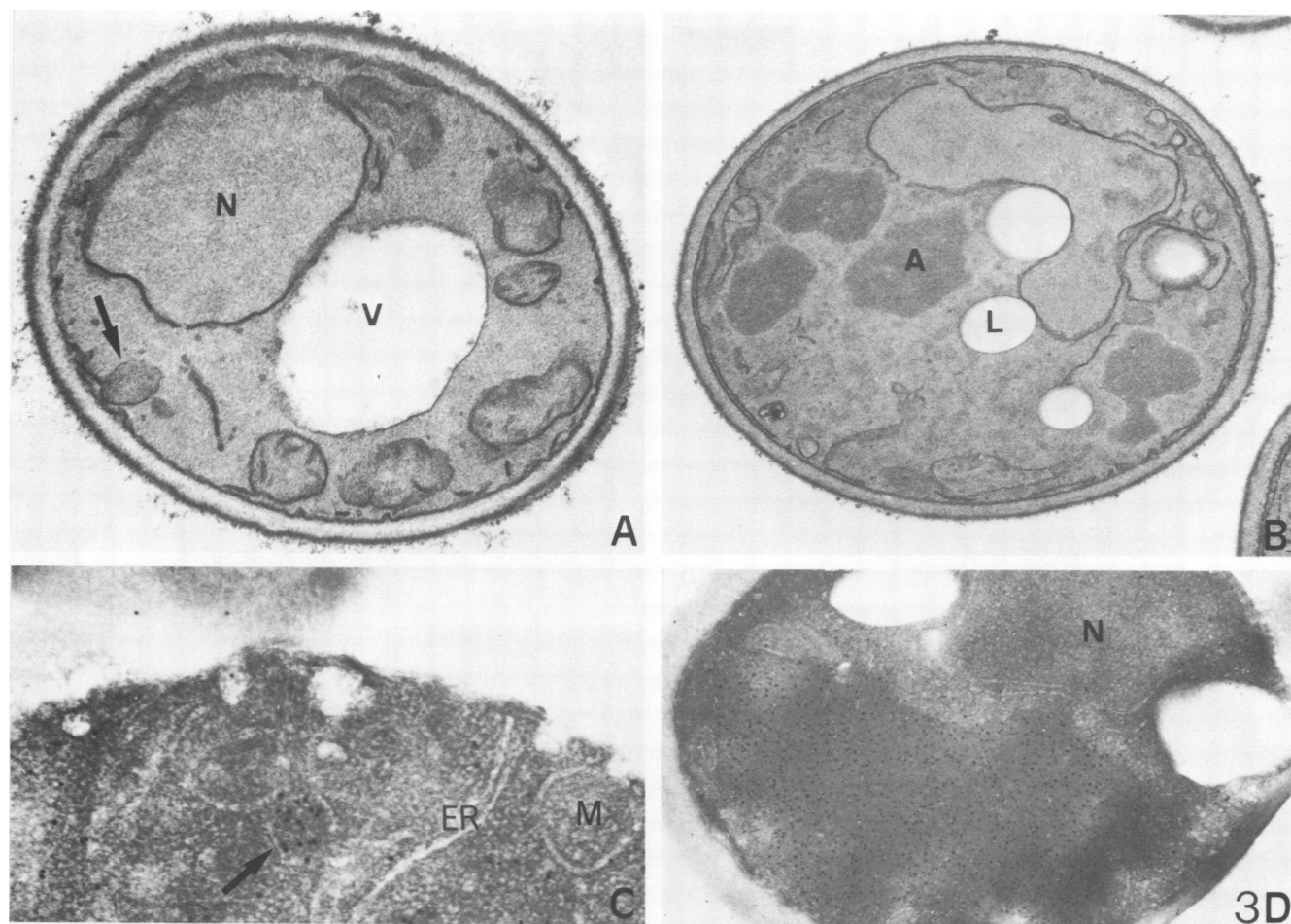
Fig. 3. Electron micrographs of transformed and control cells grown on ethanol. (**A**) Overall cell morphology of a control cell; the typical peroxisomal profile of such cells is indicated by the arrow (×28 000). (**B**) Thin section of a p40K-MOX51/2μ transformed cell showing the presence of electron dense aggregates (×22 500). (**C**) Detail of a p40K-MOX51/CEN transformed cell showing labeling on the peroxisomal profile and in the cytoplasm after staining of alcohol oxidase protein by the protein A/gold method (×55 000). (**D**) p40K-MOX51/2μ transformed cell showing intense labeling of the electron dense aggregates [staining as in (C), ×30 000]. For techniques used in electron microscopy see Veenhuis *et al.* (1976) and Douma *et al.* (1985). A, proteinaceous inclusion; ER, endoplasmic reticulum; L, lipoid droplet; M, mitochondrion; N, nucleus; V, vacuole.

Lack of enzyme activity and efficient oligomerization

Attempts to demonstrate alcohol oxidase activity in transformed *S. cerevisiae* cells have been unsuccessful so far. Alcohol oxidase activity was not present in cell-free extracts and could not be detected in whole cells by cytochemical means.

To test whether the absence of alcohol oxidase activity was due to an inefficient oligomerization of the protein, lysates of p40K-MOX51 transformed cells were subjected to velocity gradient sedimentation in sucrose gradients as described by Goodman *et al.* (1984). The fractions obtained were analysed by SDS−polyacrylamide gel electrophoresis and Western blotting (Figure 6). Alcohol oxidase protein was detected at the top of the gradient (fraction 8) at the position typical for a protein with a molecular mass of monomeric alcohol oxidase. In a control gradient purified alcohol oxidase (octamer) migrated to fraction 4. These results suggest that our inability to detect enzyme activity may be related to a lack of efficient octamerization of subunits into a functional enzyme.

Discussion

We have introduced the *H. polymorpha* gene coding for alcohol oxidase into *S. cerevisiae* and studied its expression and the subcellular location of its product. Alcohol oxidase is one of a
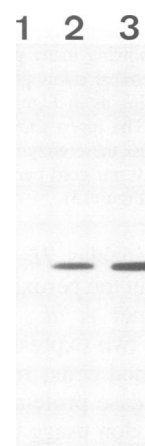

Fig. 4. Alcohol oxidase expression in oleic acid grown cells. p40K-MOX51/CEN transformed cells were grown on glucose to early exponential growth phase and subsequently inoculated in a medium with 0.1% oleic acid as carbon source. At different time points after the shift samples were withdrawn for preparation of protein extracts. Equal amounts of protein were subjected to SDS−polyacrylamide gel electrophoresis and Western blotting as described in Figure 2B. **Lane 1**, glucose grown cells (t = 0); **lane 2**, 13 h after transfer to oleic acid (t = 13); **lane 3**, 19 h after transfer (t = 19).
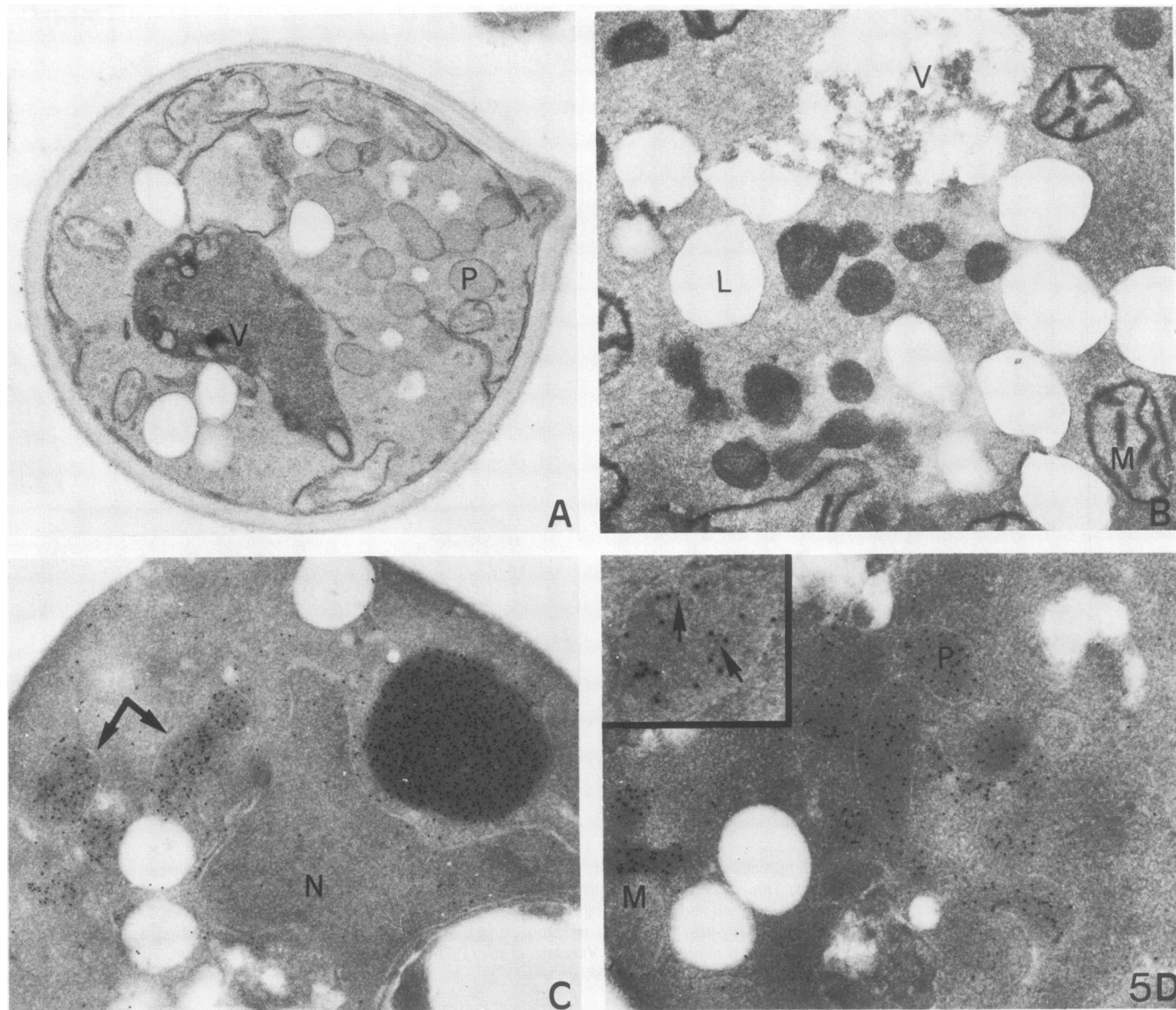
Fig. 5. Electron micrographs of transformed and control cells grown on oleic acid. (A) Overall cell morphology of a p40K-MOX51/CEN transformed cell grown for 19 h on oleic acid showing many peroxisomal profiles (×21 500). (B) Detail of a cell showing cytochemical staining of peroxisomes for catalase (×62 000). (C) Immunocytochemical staining of alcohol oxidase protein in p40K-MOX51/2μ transformed cells showing labeling of peroxisomes (arrows) and electron dense aggregates (staining as in Figure 3C, ×35 000). (D) p40K-MOX51/CEN transformed cell showing labeling on the peroxisomal profiles [staining as in (C), ×51 000]. The insert shows a detail of a p40K-MOX51/CEN transformed cell after double immunolabeling of alcohol oxidase and bifunctional enzyme. Antibodies against these enzymes were conjugated with protein A and gold particles of different diameter [alcohol oxidase: 5 nm gold particles (arrow), bifunctional enzyme: 10 nm gold particles]. For techniques used in electron microscopy see Veenhuis *et al.* (1976) and Douma *et al.* (1985). P, peroxisome (and those used in Figure 3).

group of enzymes that enables *H. polymorpha* to grow on methanol and it is located in the peroxisomes of this yeast strain. The cloned gene (Ledeboer *et al.*, 1985) was put behind a *S. cerevisiae* promoter in two expression vectors that differ in copy number in transformed cells, resulting in various steady state levels of alcohol oxidase protein per cell. Although there are some differences in codon usage between *S. cerevisiae* and *H. polymorpha* (Ledeboer *et al.*, 1985; Janowicz *et al.*, 1985), the alcohol oxidase gene contains most of the consensus sequence needed for efficient initiation of translation (Kozak, 1986), and a Zaret and Sherman sequence (1982) which is believed to function as transcription termination or polyadenylation signal in *S. cerevisiae*. The high level of expression obtained suggests that these signals are efficiently recognized, although specific mRNA levels were not determined in the transformed cells.

We have demonstrated by immunocytochemistry that alcohol oxidase, synthesized in the cytoplasm, is imported into peroxisomes indicating that the heterologous import signal present in the alcohol oxidase protein is efficiently recognized in *S. cerevisiae*. Import is not conditional since, both under uninduced conditions in which cells contain only one or a few peroxisomes (ethanol grown cells) as well as under induced conditions with many peroxisomes per cell (oleic acid grown cells), alcohol oxidase is routed to the peroxisomes.

Although the protein reaches the organelle in which it is normally active in *H. polymorpha*, we have not been able to detect alcohol oxidase activity in transformed *S. cerevisiae* cells. Several alternatives can be considered to explain this result. First, we cannot exclude the remote possibility that amino acids from N- or C-terminal end(s) might be missing, even though the alcohol
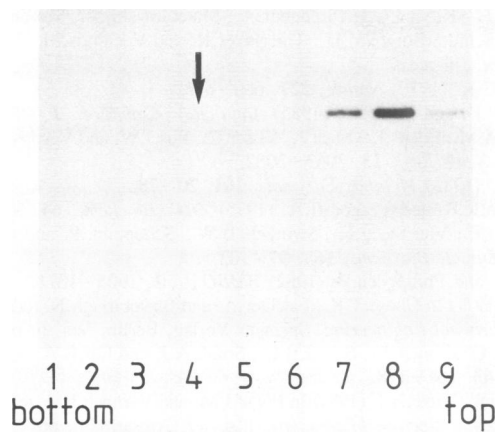
Fig. 6. Sucrose gradient centrifugation of yeast lysates. p40K-MOX51/CEN transformed cells grown on oleic acid as carbon source were lysed in a buffer containing 1% Triton-X-100 as described under Materials and methods. The lysate was centrifuged for 20 min at 15 000 g and the supernatant applied to a 5−20% (w/v) sucrose gradient. After centrifugation (55 000 r.p.m., 3.5 h, 2°C) fractions were collected from the bottom. Protein present in these fractions was subjected to SDS−polyacrylamide gel electrophoresis and treated as in Figure 2B. Purified alcohol oxidase (octamer) and bovine serum albumin were run on a parallel gradient. BSA (68 kd) used as a marker for monomeric alcohol oxidase (73 kd) migrated to fraction 8, alcohol oxidase octamers were found in fraction 4 (indicated by the arrow).

oxidase in cell-free protein extracts from transformed cells is indistinguishable in size from the fully active enzyme purified from H. polymorpha in SDS−polyacrylamide gels. Second, alcohol oxidase contains flavine adenine dinucleotide (FAD) as a prosthetic group. A limitation in availability of FAD due to overproduction of alcohol oxidase seems rather unlikely since transformed cells are not noticeably handicapped in growth rate compared to untransformed cells, and addition of FAD to the growth medium has no effect on the amount of alcohol oxidase activity found. Furthermore, acyl CoA oxidase, a FAD-containing peroxisomal protein, is induced and active when S. cerevisiae is grown on oleic acid (Kunau et al., 1987), conditions in which no alcohol oxidase activity can be demonstrated either. Third, peroxisomal proteins are almost without exception multimeric proteins assembled from identical subunits. Sucrose gradient sedimentation analysis indicates that the imported alcohol oxidase does not oligomerize into octamers, the active form of the enzyme in H. polymorpha. The presence of alcohol oxidase monomers in the peroxisomes of S. cerevisiae suggests that import precedes complex formation. Peroxisomes of methanol-grown H. polymorpha cells contain a crystalline matrix and it has been demonstrated that alcohol oxidase is the main structural component of these crystalloids (Veenhuis et al., 1981). It could be argued that such higher order structure is achieved with the help of other protein(s), comparable in function to the 'binding' proteins that are necessary for the assembly of the enzyme ribulose biphosphate carboxylase (Cannon et al., 1986). This is, however, unlikely on the basis of recent unpublished experiments (Distel et al., in preparation). We have introduced an extra copy of the alcohol oxidase gene in H. polymorpha which is constitutively expressed. This constitutively synthesized protein is imported in the peroxisome(s), is enzymatically active and gives rise to a crystalline substructure of the peroxisomal matrix under conditions in which endogenous genes, the products of which permit assimilation of methanol, are completely shut off. Such crystalloids have never been observed in transformed

S. cerevisiae cells. There must therefore exist a difference in the peroxisomal matrix milieu between H. polymorpha and S. cerevisiae that is responsible for the lack of octamerization in S. cerevisiae peroxisomes. By investigating the import of another multimeric peroxisomal protein from H. polymorpha we can establish if this is a phenomenon peculiar of alcohol oxidase or a general property of heterologous protein import into peroxisomes.

An interesting implication of our results is that a heterologous signal for import into peroxisomes is recognized in S. cerevisiae. Together with the recent discovery that peroxisomes can be induced in S. cerevisiae, the model system described in this paper allows us to identify the nature of peroxisomal import signal(s) in an organism which is very suitable for studies on protein targeting.

Materials and methods

Strains, culture conditions and plasmids

Strains used in this study were S. cerevisiae DL1 (α, leu2-3, 2-112, his3-11, 3-15, ura3-251, 3-372, 3-328) (Van Loon et al., 1983) BJ1991)α, leu2, trp1, ura3-52 prb1-1122, pep4-3 (Jones, 1977) and Escherichia coli JM101 [supE, thi, Δ(lac-proAB),(F′, traD36, proAB, lacIq ZΔ M15)] (Messing, 1983). E. coli cells were grown in 2YT medium in the presence or absence of 50 μg/ml ampicillin. Yeast transformants were selected on agar plates containing 0.67% yeast nitrogen base without amino acids (WO) (Difco), 2% glucose and amino acids (20 μg/ml) as needed. Individual transformants were grown, after preselection in minimal medium, in WOYE (WO medium plus 0.1% yeast extract and 1% ethanol) and harvested in the exponential growth phase. For transfer experiments to oleic acid containing medium yeast transformants were precultured in WOY with 0.3% glucose to the early exponential growth phase (A600 = 1.0) and subsequently diluted (1:10) in WOY plus 0.1% oleic acid and 0.1% Tween-40. Plasmid loss after growth on non-selective medium was determined in each experiment by plating dilutions of the culture on selective and non-selective agar plates. Plasmid pEMBL Ye23R is identical to pEMBL Ye23 (Baldari and Cesareni, 1985) except that the EcoRI site in the 2 μm region has been removed. Plasmid pUC18 and pLA433 have been described (Vieira and Messing, 1982; Panzeri and Philippsen, 1982).

Construction of the p40K-MOX51 plasmids

A 5 kb BamHI−SacI fragment containing the entire alcohol oxidase structural gene including the 5′- and 3′-flanking sequences (Ledeboer et al., 1985) was cloned in the polylinker of plasmid pUC18 (pMOX-BS) (Figure 1). Most of the 5′-untranslated sequences were removed by Bal31 digestion and suitable clones were selected by restriction enzyme and DNA sequence analysis. The clone pMOX51 which extends to position −10 was fused with a fragment containing the promoter of the 40 kd subunit of complex III. The DNA construct was cloned in a multi-copy plasmid Ye23R (p40K-MOX51/2μ). The p40K-MOX51/CEN plasmid was constructed by isolating the HindIII/SmaI fragment (containing the 40K promoter) from pYe23R-40K. The HindIII site was filled in with Klenow DNA polymerase and the fragment cloned in the single-copy centromere containing plasmid pLA433 opened at its BamHI site (filled in with Klenow DNA polymerase). The unique BamHI site in this plasmid (at the 3′-end of the 40K promoter fragment) was used to insert the MOX51 BamHI fragment isolated from p40K-MOX51.

Preparation of protein extracts

Yeast proteins from SDS−polyacrylamide gel electrophoresis were isolated by breaking the cells with glass beads and acid precipitation as described by Needleman and Tzagoloff (1975). For enzyme assays the cells were lysed in 50 mM potassium phosphate buffer, pH 7.4 and 1 mM phenyl methyl sulfonyl fluoride (PMSF) using glass beads. Cell debris was removed by centrifugation at 15 000 g for 20 min. In cells transformed with the multi-copy recombinant plasmid (p40K-MOX51/2μ) ∼80% of the alcohol oxidase protein was associated with the pellet, whereas in cells transformed with the single-copy recombinant plasmid (p40K-MOX51/CEN) only 20% was found in the 15 000 g pellet. In the presence of 1% Triton X-100 in the lysis buffer these values were 40% and 3% respectively. Removing the cell wall before lysis had no effect on the amount of alcohol oxidase protein found in the pellet. The alcohol oxidase associated with the 15 000 g pellet probably represents aggregates of monomers (see Results).

Separation of monomeric and octameric alcohol oxidase

Cells were broken in lysis buffer (10 mM Tris/HCl, pH 8.0, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 1 mM PMSF) with glass beads and after removing the cell debris at 15 000 g samples were separated on 5−20% sucrose gra-

dients according to Goodman *et al.* (1984). Fractions were analysed by SDS−polyacrylamide gel electrophoresis (Laemmli, 1970) and Western blotting (Vaessen *et al.*, 1981).

Purification of alcohol oxidase and preparation of antiserum

H. polymorpha cells (strain CBS 4732) grown in a methanol limited chemostat were lysed in 50 mM potassium phosphate buffer, pH 7.5 and 1 mM PMSF with glass beads. Alcohol oxidase was purified by ammonium sulphate precipitation (Douma *et al.*, 1985). The fraction precipitating at 45% saturation contained most of the activity and was subjected to Sephacryl S300 gel filtration in 100 mM potassium phosphate, pH 7.5/0.2 mM PMSF. The peak fractions were loaded onto a 5.5% polyacrylamide slab gel (in which SDS was omitted) and electrophoresed at 8 mA as described by Goodman (1984). The yellow band representing the flavoprotein was cut out of the gel and eluted overnight at 4°C in 50 mM phosphate buffer. The purified protein was denatured by boiling in SDS and used to generate antiserum in a New Zealand white rabbit by subcutaneous injections.

Enzyme assays

Alcohol oxidase activity in crude extracts and purified fractions was measured spectrophotometrically at 420 nm in 50 mM potassium phosphate buffer pH 7.0, 10 U/ml peroxidase, 0.5 mg/ml ABTS [2,2-azino-di-(3-ethyl-benzthiazolin-sulfonate)] and 120 mM methanol. Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as a standard.

Electron microscopy, cytochemistry and immunocytochemistry

Whole cells were fixed in $KMnO_4$ for 20 min at room temperature, spheroplasts were fixed in glutaraldehyde/OsO_4. After dehydration in a graded ethanol series the cells were embedded in Epon 812 by the methods described by Douma *et al.* (1985). For immunocytochemistry intact cells were fixed in 3% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.2 for 60 min at 0°C, dehydrated in ethanol and embedded in Lowicryl K4M (Zagers *et al.*, 1986). Polymerization was at −35°C with u.v. light. Protein A/gold labeling was performed on ultrathin sections of K4M-embedded cells by the method of Slot and Geuze (1984). The double immuno-detection method using two antibodies each marked with different sized gold particles was carried out according to Douma *et al.* (1985). Cytochemical staining for the detection and localization of catalase was performed as described previously (Veenhuis *et al.*, 1976).

Other procedures

Published procedures were used for yeast transformation (Klebe *et al.*, 1983), plasmid isolation (Birnboim and Doly, 1979), preparation of spheroplasts (Daum *et al.*, 1982) and sequence analysis (Sanger *et al.*, 1980).

Acknowledgements

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