Alcohol Oxidase Expressed under Nonmethylotrophic Conditions Is Imported, Assembled, and Enzymatically Active in Peroxisomes of *Hansenula polymorpha*

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**Abstract.** We have introduced into *Hansenula polymorpha* an extra copy of its alcohol oxidase gene. This gene which is under the control of the *Saccharomyces cerevisiae* phosphoglycerate kinase promoter is integrated in a chromosome different from the one containing the endogenous gene. Cells with the extra alcohol oxidase gene, grown on glucose or ethanol as the sole carbon source, express enzymatically active alcohol oxidase. However, other enzymes characteristic for methylotrophic growth conditions are absent or present at low levels. Most of the alcohol oxidase occurs in the octameric state and immuno- and cytochemical evidence shows that it is located in a single enlarged peroxisome per cell. Such peroxisomes show crystalloid inclusions which are lacking in the peroxisomes present in glucose grown control cells.

Our results suggest that import into peroxisomes of *H. polymorpha*, assembly and activation of alcohol oxidase is not conditionally dependent on adaptation to methylotrophic growth conditions and that proliferation of peroxisomes is a well-programmed process that is not triggered solely by overproduction of a peroxisomal protein.

**Eukaryotic** cells contain a number of membrane-enclosed compartments each devoted to carry out specific metabolic functions. To this purpose each organelle possesses its own characteristic group of enzymes and proteins. Some of these organelles have been favorite objects of research and much is already known about their contribution to cellular metabolism and biogenesis. Relatively little is known however, about peroxisomes, which were first discovered by De Duve et al. (1966). Although rather variable in enzymatic repertoire depending on the organism or tissue in which they occur, a common feature is the presence of an H$_2$O$_2$ producing oxidase and catalase. Recent studies have revealed that peroxisomal proteins, including integral membrane proteins, are encoded in the nucleus, synthesized on free polysomes and imported posttranslationally into the organelle (reviewed by Lazarow and Fujiki, 1985). Moreover, the majority of peroxisomal proteins are synthesized at their mature size and translocation across the peroxisomal membrane occurs without any detectable modification of the protein (reviewed by Borst, 1986).

A very interesting example of peroxisomal function and development is represented by methylotrophic yeasts. In these fungi the number of organelles and their physiological function is entirely dependent upon environmental conditions (for reviews see Veenhuis et al., 1983 and Veenhuis and Harder, 1987). For instance when the methylotrophic yeast *Hansenula polymorpha* is grown on methanol the cells contain a large number of peroxisomes which have a crystalline matrix exclusively composed of alcohol oxidase (AO) molecules (Veenhuis et al., 1978; Veenhuis et al., 1981). AO functions as the first enzyme in methanol metabolism converting methanol to formaldehyde and H$_2$O$_2$. Besides AO considerable amounts of catalase and dihydroxy acetone synthase are present in these organelles (Douma et al., 1985; Goodman, 1985). Catalase is indispensable in removing the potentially toxic H$_2$O$_2$ and dihydroxy acetone synthase is the first enzyme in the methanol assimilatory pathway. In contrast, during exponential growth of *H. polymorpha* on glucose the synthesis of these enzymes is repressed (Eggeling and Sahm, 1978; Egli et al., 1980) and the cells generally contain a single small peroxisome located in close proximity to the cell wall (Veenhuis et al., 1979). Thus, methylotrophic yeasts offer an attractive model system to study peroxisome biogenesis and to address questions relating to import of proteins into these organelles.

Studies on routing of AO have revealed that inactive protein monomers are synthesized in the cytoplasm (Roa and Blobel, 1983; Roggenkamp, 1984). These subunits assemble within a few minutes after synthesis into the active (octamer-
meric) form of the enzyme which is found only in the organelle (Goodman et al., 1984). It has been observed that in the early phase of adaptation to methanol protein monomers temporarily accumulate, while during later stages such a pool of subunits is much smaller (Brooke, A. G., personal communication). Moreover, studies on peroxisomal membrane formation under similar conditions have shown that specific membrane proteins are induced (Goodman et al., 1986). Whether this involves synthesis of new membrane proteins or increased production of pre-existing proteins could not be inferred from this work due to the limit of detection of peroxisomal membrane proteins in glucose grown cells. Both observations suggest however that peroxisomes may require membrane components (for instance receptors) before they can take up the new set of proteins prescribed by the change of carbon source in the growth medium. To study these aspects of peroxisomal protein import in more detail we have constructed by genetic manipulation a strain of *H. polymorpha* in which AO is produced in the absence of methanol. During exponential growth of this transformant on glucose or ethanol, AO is efficiently routed into the single peroxisome present in these cells. Moreover, the imported AO assembles into the enzymatically active holoenzyme giving rise to a crystalline inclusion within the organelle.

Materials and Methods

Strains, Culture Conditions, and Plasmids

Strains used in this study were *H. polymorpha* (leu) obtained from Dr. P. E. Sudbery (Gleeson et al., 1986) and *Escherichia coli* 490 (recA hsdR17 hsdM17). Complex medium for growth of yeast was YPG (1% yeast extract, 2% peptone, 2% glucose). Minimal medium for yeast contained 0.67% yeast nitrogen base without amino acids and 2% glucose or 0.5% methanol. In some experiments minimal batch medium was used as described by Van Dijken et al. (1976). Required amino acids were added at a concentration of 20 μg/ml. *H. polymorpha* cells were grown in all experiments at 37°C. *E. coli* cells were grown on 2YT (1% yeast extract, 1.6% tryptone, 0.5% sodium chloride) supplemented with 50 μg/ml ampicillin when necessary. Plasmid YEp3, containing the LEU2 gene of *Saccharomyces cerevisiae* as selective marker (Broach et al., 1979) was used for transformation of *H. polymorpha* (leu). The yeast expression vector pMA91 (Mellor et al., 1983) was obtained from Dr. S. M. Kingsman.

Construction of the pPGK-MOX51 Plasmid

The final construct as outlined in Fig. 1 was obtained by insertion of the 2.4-kb Bam HI fragment of p40K-MOX51/2 (Distel et al., 1987), containing the entire AO coding sequence, into the Bam HI site of the yeast expression vector pMA91. The 4.3-kb Hind III fragment, comprising the AO coding sequence in the correct orientation sandwiched between the 5' and 3' flanking sequences of the PGK gene, was cloned into the Bam HI site of YEp3 after Bam HI linker addition.

Yeast Transformation

*H. polymorpha* cells were transformed according to the method described by Klebe et al. (1983), using frozen cells treated with polyethylene glycol, with the exception that incubations were done at 37°C.

DNA Preparation

Total DNA was isolated from yeast as described by Holm et al. (1986) starting with 20-ml cultures grown to the early logarithmic phase. DNA isolated by this method contains both plasmid DNAs and largely intact genomic DNA and was used for transformation of *E. coli* and Southern blot analysis. Isolation of plasmid DNA from *E. coli* was performed by the alkaline lysis method (Birnboim and Doly, 1979).

Southern Blot Experiments

For Southern blots (Southern, 1975), the yeast DNA was digested for 3 h with appropriate restriction enzymes and the digests were separated on 0.7% agarose gels. After transfer of the DNA to nitrocellulose the blots were hybridized with 32P nick translated probes. The following fragments were used as hybridization probes: 2.5-kb Sal I-Sal I fragment derived from the 5'-end of the AO gene (3'AO probe), 1.85-kb Xho I-Sac I fragment from the 3'-end of the AO gene (3'AO probe) (Ledeboer et al., 1985), 1.5-kb Bgl II-Hind III fragment of plasmid PMA91 (Mellor et al., 1983) containing the PGK promoter (PGK probe) and the 2.3-kb Sal I-Xho I fragment of YEp3 (Broach et al., 1979) containing the LEU2 gene (LEU probe).

Cell-free Extract Preparation and Enzyme Assays

Cell free extracts for determination of enzyme activities were prepared as described by De Koning et al. (1987). Enzyme assays were carried out at 37°C. AO was assayed according to Distel et al. (1987) using chromogenic substrate. Formaldehyde dehydrogenase and formate dehydrogenase were determined according to Van Dijken et al. (1976), dihydroxyacetone synthase according to Waets and Quayla (1981). Catalase was assayed as described by Lück (1963). Protein concentrations in cell-free extracts were determined by the method of Bradford (1976) using BSA as a standard.

Electron Microscopy, Cytochemistry, Immunochemistry

Whole cells were fixed in 1.5% KMnO4 for 20 min at room temperature. Spheroplasts were fixed in 6% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 0°C, followed by post fixation in a 1:1 mixture of 2% OsO4 and 5% K2Cr2O7 in the same buffer for 90 min at 0°C. After dehydration in a graded ethanol series the material was embedded in Epon812. Cytochemical staining of AO was performed on glutaraldehyde fixed spheroplasts, using the CeCl3 method (Veenthuis et al., 1978). Protein A-gold labeling of AO was performed on thin sections of Lowicryl K4M-embedded intact cells as described by Douma et al. (1985).

Other Methods

Yeast DNA for pulse-field gradient electrophoresis was prepared in agarose blocks and separated on 0.5% agarose gels for 24 h at 200 V with a pulse time of 3 min (Van Ommen and Verkerk, 1986). Yeast proteins prepared as described by Needleman and Tzagoloff (1975), were separated on SDS-polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose according to Vaesens et al. (1981). Separation of AO monomers and octamers was performed as described before (Goodman et al., 1984). Preparation of antibodies against AO and dihydroxyacetone synthase has been described (Distel et al., 1987; Douma et al., 1985).

Results

Construction of a *H. polymorpha* Strain With an Extra AO Gene

To obtain production of AO in *H. polymorpha* under non-methylotrophic conditions, we transformed cells with an extra copy of the AO gene after replacing its 5' and 3' flanking control regions by those of the S. cerevisiae gene coding for phosphoglycerate kinase (PGK). The PGK control regions are recognized in *H. polymorpha* and result in fairly high expression when cells are grown on glucose (see below). The reconstructed gene unit was then inserted into the YEp3 vector (Fig. 1) (for details of this construction [pPGK-MOX51] see Materials and Methods).

The recombinant plasmid pPGK-MOX51 and the YEp3 vector were each introduced into *H. polymorpha* (leu) (Gleeson et al., 1986) and the stability of the plasmids in the transformed strains was studied (Table I). When YEp3-transformed cells were grown under nonselective conditions the leu+ phenotype was quickly lost. Analysis of unrestricted DNA, isolated from cells grown under selective conditions,
by Southern blot hybridization with labeled YEpl3 revealed two distinct bands corresponding to open and closed circular plasmid monomer (not shown). These results indicate that the YEpl3 plasmid is maintained as an autonomous replicating element in H. polymorpha. Transformation of H. polymorpha with the plasmid pPGK-MOX51 leads however to integration in the nuclear genome. In Southern blots of unrestricted H. polymorpha DNA the YEpl3 probe hybridized only with the nuclear DNA and no evidence for the presence of the free plasmid forms was found (not shown). Integration did not however result in a stable phenotype, because 50% of the cells lost the leu+ phenotype when they were grown for 30 generations under nonselective conditions.

To determine the position of plasmid integration in the nuclear genome of the strain selected for further work, DNA fragmented with various restriction endonucleases was analysed by Southern blotting. The following DNA probes were used for the different parts of the recombinant DNA plasmid: 5'- and 3'-half of the AO coding sequence, DNA from the LEU2 gene and DNA of the PGK promoter region. An example of these hybridization experiments is shown in Fig. 2. DNA from transformed cells yields extra restriction DNA fragments not present in the DNA of untransformed control cells. Detailed analysis of the length of the hybridizing fragments revealed that no gross rearrangements had occurred during integration of the plasmid. In no case evidence was found for linkage between DNA fragments obtained from the extra inserted AO gene and the endogenous AO gene, suggesting that integration had not taken place in or around the endogenous AO gene itself. This was confirmed by separation of chromosomes by pulsed field gradient electrophoresis followed by Southern blot analysis of the DNA (Fig. 3). The endogenous AO gene is present on a relatively small chromosome well separated from the others. The pPGK-MOX51 DNA copy has integrated in a different chromosome too large to enter the agarose gel, as indicated by the specific hybridization with the DNA in the slot of the gel with the pPGK-MOX51 specific probes. The strong hybridization with the DNA in the slot of the gel suggests that multiple copies of the AO gene construct have been inserted into large chromosomes. However in Southern blot experiments with restricted DNA (Fig. 2) the fragments derived from the endogenous AO gene and the inserted gene were always present in stoichiometric amounts indicating that only a single copy has been integrated. The discrepancy found in these experiments may be due to trapping of the small chromosome with the endogenous AO gene in the slot of the gel. Our conclusion from these experiments is that the cis-acting control elements of the endogenous AO gene will have negligible or no influence on the expression of the extra AO gene copy we have introduced.

Expression of AO in Glucose-grown Transformed Cells

H. polymorpha transformants and control cells were grown on minimal medium with 2% glucose as the sole carbon source to early exponential phase to ensure repression of the

Table I. Properties of Plasmids in H. polymorpha

| Plasmid    | Stability* | Generations as autonomous element† |
|------------|------------|-----------------------------------|
| YEpl13     | 1          | >25                               |
| pPGK-MOX51 | 46         | <10                               |

* Expressed as percentage of remaining leucine prototrophs after growth on complete medium (YPG) for 30 generations.
† Transformants were grown logarithmically in selective medium and the state of the plasmid sequences (autonomous vs. integrated) was determined at different time points by Southern blot hybridization of labeled YEpl3 to undigested yeast DNA.
Expression of the extra copy of the alcohol oxidase gene in glucose grown *H. polymorpha* cells. Cells were cultured in minimal medium with 2% glucose as a carbon source to the early exponential growth phase (OD$_{600}$ = 1.0-1.5). Protein extracts were prepared, subjected to SDS-PAGE and transferred to nitrocellulose. Blots were incubated with anti-AO antibody and 125I-labeled protein A. (Lane 1) pPGK-MOX51-transformed *H. polymorpha* cells; (lane 2) untransformed *H. polymorpha* cells; (lane 3) purified AO from *H. polymorpha*.

Figure 3. Size-fractionation of chromosome-sized DNAs of untransformed and pPGK-MOX51-transformed *H. polymorpha* cells. Cells were gently lysed in agarose blocks and whole chromosomes were size-separated with pulsed field gradient gel electrophoresis as described in Materials and Methods. After transfer to nitrocellulose the filters were hybridized with probes specific for the PGK promoter (PGK), the AO gene (SAO) and the LEU2 gene (LEU). (Lanes 1, 3, and 4) pPGK-MOX51 integrant; (lane 2) untransformed control cells.

Table II. Enzyme Activities in Cell-free Extracts of Untransformed and pPGK-MOX51-transformed *H. polymorpha* Cells

| *H. polymorpha* cells | Carbon source | Alcohol oxidase | Dihydroxyacetone synthase | Formaldehyde dehydrogenase | Formate dehydrogenase | Catalase |
|-----------------------|--------------|----------------|--------------------------|---------------------------|----------------------|---------|
| Untransformed         | Glucose      | <0.5           | <1                       | 145                       | <0.5                 | 2.4     |
| Transformed           | Glucose      | 184            | <1                       | 149                       | <0.5                 | 3.1     |
| Transformed           | Methanol     | 571            | 78                       | 631                       | 353                  | 39      |

*H. polymorpha* cells grown on minimal medium with 2% glucose or 0.5% methanol as the sole carbon source were harvested in the early logarithmic phase (OD$_{600}$ = 1.0) and protein extracts were prepared as described by De Koning et al. (1987).

Enzyme activities are expressed as nmol/min$^{-1}$ per mg$^{-1}$ except for catalase which is expressed as ΔE$_{405}$/min$^{-1}$ per mg$^{-1}$.

Subcellular Location of AO

The overall cell morphology of glucose-grown untransformed cells (not shown). The presence of the extra AO gene does not disturb the response of the transformed cells in their adaptation to methanol. These results indicate that proteins characteristic for the methylotrophic state are absent in the glucose-grown transformed cells and suggest that the AO is expressed from the introduced gene. The AO is in all respects similar to the enzyme expressed from the endogenous gene under methylotrophic conditions. This is supported by further controls.

Protein extracts of cells were fractionated by SDS-PAGE and proteins were transferred to nitrocellulose for immuno-detection (Fig. 4). In extracts of pPGK-MOX51-transformed cells a single polypeptide was found that reacts with AO-specific antiserum and co-migrates with authentic AO protein. In untransformed cells grown under identical conditions no AO protein could be detected.

To investigate whether AO monomers are assembled into octamers, we subjected protein extracts to velocity sedimentation on sucrose gradients. Proteins in collected fractions were analyzed by SDS-PAGE and AO was detected by Western blot analysis (Fig. 5). Most of the AO (93%) of transformed cells sediments at a position identical to that of purified (octameric) AO used as a marker. Only a small amount (7%) remains near the top of the gradient.

We propose that the introduced AO gene is actively transcribed from the *S. cerevisiae* PGK promoter in cells grown on glucose as carbon source and that the expressed protein is indistinguishable from the endogenous AO produced under methylotrophic conditions. Similar results were obtained with ethanol as the sole carbon source (I. van der Klei, unpublished experiments).
formed cells is shown in Fig. 6 A. Apart from the usual cell organelles such as nuclei, mitochondria and vacuoles these cells contain a single microbody (arrow) which is a characteristic phenomenon for such cells (Veenhuis et al., 1979). However, in transformed cells grown under the same conditions, considerably enlarged peroxisomes (up to 0.5 μm in diameter) were present (Fig. 6 B). Morphometric analysis revealed that in these cells the peroxisomal volume fraction (expressed as percentage of the cytoplasmic volume) was 3.6% whereas in control cells a value of 0.1% was found. Also the transformed cells generally contained only one organelle and proliferation of peroxisomes was not observed. The presence of a single organelle in these cells was substantiated by immunofluorescence microscopy (data not shown). The peroxisomal matrix harbour large crystalline inclusions (Fig. 6 D) which were lacking in control cells (Fig. 6 C). Cytocchemical experiments revealed that these peroxisomes were the sole sites of AO activity in transformed cells (Fig. 6 E); therefore the crystalloids most probably result from crystallization of AO protein (Veenhuis et al., 1981). The presence of AO protein in the peroxisomal matrix was confirmed immunocytochemically (Fig. 6 F). These experiments furthermore indicated that the bulk of the protein was efficiently targeted into peroxisomes since cytosolic labeling after protein A-gold incubation was largely lacking.

**Discussion**

We have equipped a *H. polymorpha* strain with an extra gene coding for alcohol oxidase under the direction of the *S. cerevisiae* phosphoglycerate kinase promoter. Southern blot analysis revealed that the entire plasmid with the gene construct has been integrated into the genome. Furthermore, we showed by pulsed field gradient electrophoresis experiments that the endogenous AO gene and the newly introduced gene were present on different chromosomes. The different chro-

mosomal location of the AO genes ensures that expression of the introduced gene copy is not under the cis-acting control elements belonging to the endogenous gene. Indeed, when this strain is grown on glucose (or ethanol) as a carbon source, AO is constitutively produced while genes coding for proteins characteristic for the methylotrophic state remain switched off. The AO produced under these growth conditions is in all respects identical to the protein derived from the endogenous gene. Subunits have a similar molecular weight, the enzyme occurs in octameric state, is enzymatically active and is assembled into typical crystalloid structures.

Preliminary results suggested that peroxisomes in cells transferred to methylotrophic growth conditions may require adaptation before the new set of proteins can be imported. This was mainly based on the observation that monomeric AO accumulates in the first hours after the shift from glucose to methylotrophic growth conditions, while in fully adapted cells such a subunit pool is much smaller. Our results indicate however, that the peroxisomal import system for AO is constitutively present and active since AO is efficiently routed into peroxisomes of glucose-grown cells. Since key enzymes characteristic for methylotrophic growth are absent or present at only very low levels there seems to be no need for prior association of enzyme complexes at the peroxisomal membrane before successful import can take place as suggested by Bellion and Goodman (1987). We can however, not exclude the possibility that another protein(s) is required to assemble AO into octamers and/or organize these into a crystalloid structure. Proteins with such a function have been described earlier and called "molecular chaperones" because they help polypeptide chains to fold and assemble into oligomeric structures (Ellis, 1987). If present in *H. polymorpha* the results obtained suggest that this protein(s) is constitutively expressed. In *S. cerevisiae* expression of AO results in import of subunits into peroxisomes, but association of monomers into octamers and formation of the active holo-enzyme does not take place (Distel et al., 1987). Absence of a suitable "chaperone" protein in this yeast could be one of the explanations for this observation.

The peroxisomes in glucose-grown transformed cells are considerably enlarged, compared with those present in untransformed control cells, grown under similar growth conditions. Apparently, the extra import of a peroxisomal protein (AO) is also associated with the incorporation of phospholipids in the peroxisomal membrane, because simple stretching of the pre-existing membrane is insufficient to account for the observed increase in size of the organelle. Whether uptake of phospholipids is accompanied by the incorporation of membrane proteins is now under investigation.

In *H. polymorpha* adapted to methylotrophic growth conditions, new peroxisomes are formed by pinching off small vesicles from full grown mature peroxisomes (Veenhuis et al., 1978). This process does not take place in the transformed cells grown on glucose. It suggests that AO itself cannot act as an inducer of peroxisome proliferation and the compound(s) and/or protein(s) which trigger this process in methylotrophic yeasts remain to be discovered.

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Figure 6. Electron micrographs of transformed and control cells grown on glucose. Survey of control (A) and transformed cells (B) grown on glucose, showing the overall cell morphology. The small peroxisome in the control cells is indicated by an arrow (whole cells fixed with KMnO₄). (C) Detail of a control cell to demonstrate that the organelle did not contain crystalline inclusions which were evident in peroxisomes of transformed cells (D) (spheroplasts fixed with glutaraldehyde-OsO₄/K₃Cr₂O₇). (E) Cytochemical staining of transformed cells to demonstrate AO activity in peroxisomes (glutaraldehyde-fixed spheroplasts incubated with CeCl₃ and methanol). (F) Immunocytochemical staining of AO protein in transformed cells showing labeling on the peroxisomal profile (arrow) (glutaraldehyde-fixed whole cells embedded in Lowicryl K4M incubated with antibodies against AO and protein A-gold). Crystalloids are only visible in glutaraldehyde-fixed spheroplasts.

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