Pyruvate carboxylase is an essential protein in the assembly of yeast peroxisomal oligomeric alcohol oxidase.

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Abstract.

_Hansenula polymorpha ass3_ mutants are characterized by the accumulation of inactive alcohol oxidase (AO) monomers in the cytosol, while other peroxisomal matrix proteins are normally activated and sorted to peroxisomes. These mutants also have a glutamate or aspartate requirement on minimal media. Cloning of the corresponding gene resulted in the isolation of the _H. polymorpha PYC_ gene that encodes pyruvate carboxylase (HpPyc1p).

HpPyc1p is a cytosolic, anapleurotic enzyme that replenishes the tricarboxylic acid cycle with oxaloacetate. The absence of this enzyme can be compensated by addition of aspartate or glutamate to the growth media.

We show that HpPyc1p protein, but not the enzyme activity is essential for import and assembly of AO. Similar results were obtained in the related yeast _Pichia pastoris_.

_In vitro_ studies revealed that HpPyc1p has affinity for FAD and is capable to physically interact with AO protein. These data suggest that in methylotrophic yeast pyruvate carboxylase plays a dual role in that, besides its well-characterized metabolic function as anapleurotic enzyme, the protein fulfils a specific role in the AO sorting and assembly process, possibly by mediating FAD-binding to AO monomers.
Introduction.

The yeast *Hansenula polymorpha* is able to use methanol as sole carbon and energy source. Growth on this compound is accompanied by the induction of peroxisomes that contain the key enzymes of methanol metabolism. Alcohol oxidase (AO) is a major constituent of these organelles and catalyzes the oxidation of methanol into formaldehyde and hydrogen peroxide. Inactive AO monomers are synthesized in the cytosol and posttranslationally imported into the target organelle, where the protein is activated. The active enzyme is an octamer, containing 8 identical subunits, which each contains a flavin adenine dinucleotide (FAD) molecule as cofactor (reviewed by van der Klei et al., 1991a).

Both *in vivo* and *in vitro* experiments suggested that assembly of AO into active octamers is most likely not a spontaneous process (Distel *et al.*, 1987; van der Klei *et al.*, 1989b). Several independent experiments suggested that specific helper proteins (tentatively called assembly factors) are required to mediate AO assembly. Studies on an *H. polymorpha* riboflavin (Rf) auxotrophic mutant revealed that Rf limitation interfered with the assembly and the import of AO (Evers *et al.*, 1994) and suggested that co-factor binding, oligomerization and translocation of AO are tightly coupled processes. However, in all *H. polymorpha* peroxisome-deficient (*pex*) mutants analyzed so far AO is normally assembled and active in the cytosol. This suggests that AO assembly does not require the specific (acidic) micro-environment of the peroxisomal matrix (van der Klei *et al.*, 1991c).

Previous biochemical approaches to identify AO assembly factors failed so far. We therefore sought to isolate these components by a genetic approach. To this end we have
isolated a collection of mutants that displayed reduced AO activities (van Dijk et al., 2002).

Here, we report the functional complementation of one of these mutants. We show that the protein product of the complementing gene, pyruvate carboxylase, has a dual function in that the protein, but not the enzyme activity, is crucial for sorting and subsequent assembly of AO protein in peroxisomes of *H. polymorpha*. 
Materials and methods

Organisms and growth conditions

*Escherichia coli* strains DH5α and C600 were cultivated as described (Sambrook *et al.*, 1989). The *H. polymorpha* strains used in this study are NCYC 495 (*leu*1.1), NCYC 495 (*leu*1.1 *ura*3) (Gleeson and Sudbery, 1988) and mutants derived from these strains, *ass*3-110.*leu* 1.1 (van Dijk *et al.*, 2002), *Δpex*3 *ura*3 (Baerends *et al.*, 1996), *Δpyc*1 *leu*1.1, *Δpex*3 *Δpyc*1 and *Δpyc*1:*P*AMOPYC.

*H. polymorpha* cells were grown on minimal media containing 0.67% (w/v) Yeast Nitrogen Base without amino acids (DIFCO) containing 1% glucose (YNM) or 0.5% methanol (YNM); on YPD containing 1% yeast extract, 1% peptone and 1% glucose or mineral medium (van Dijken *et al.*, 1976) supplemented with 0.5% carbon source and 0.25 % nitrogen source. For the induction of peroxisomes mutant strains were precultivated in YPD medium and shifted to methanol containing mineral medium for 16 h. To accumulate monomeric AO in the cytosol of *Δpyc*1::*P*AMOPYC, cells were grown for 6 hours on media containing 0.1 % glycerol/0.5% methanol/0.25% ammonium sulphate. Subsequently, the cells were incubated for 30 min. in media without carbon or nitrogen sources (Waterham *et al.*, 1993) followed by transfer to mineral media containing 0.5 % glucose and 0.25 % ethylamine.

*Pichia pastoris* wild-type MP 36 (*his*3) and MP 36-*Δpyc*(pyc::*his*3) (Menendez *et al.*, 1998) were cultivated as described by (Faber *et al.*, 1998).

When needed uracil (20 mg/l), leucine (20 mg/l), histidine (40 mg/l), aspartate (60 mg/l) or glutamate (60 mg/l) were added to the media.
**Isolation and characterization of the pyruvate carboxylase (PYC1) gene**

Genetic manipulations of *H. polymorpha* were performed as described previously (Gleeson and Sudbery, 1988; Faber *et al.*, 1992; Faber *et al.*, 1994; Titorenko *et al.*, 1993). Standard recombinant DNA techniques were carried out essentially as described (Sambrook *et al.*, 1989). Endonuclease restriction enzymes and biochemicals were obtained from Roche (Almere, the Netherlands) and used as detailed by the manufacturer.

To clone the complementing genomic fragment, mutant *ass3-110* was transformed with an *H. polymorpha* genomic library (Tan *et al.*, 1995). Leucine prototrophs were tested on YNM-plates for the ability to grow on methanol (Mut⁺). Mut⁺ transformants were selected. Their plasmid content was isolated and reintroduced in mutant *ass3-110*. Four plasmids that complemented *ass3-110* again were selected for further analysis. These plasmids contained overlapping genomic fragments ranging in size from 6.5 to 9.0 kb. A 4.2-kb complementing DNA fragment was subcloned as an *EcoRI-XbaI* fragment into pBluescript II KS⁺ (pBSII KS⁺) (Stratagene Inc., San Diego, CA). Sequencing of both strands was carried out on a LiCor automated DNA-sequencer using dye-primer chemistry (LI-COR INC., Lincoln, NE). For DNA and amino acid sequence analysis, the PC-GENETM program (Release 6.70, IntelliGenetics, Mountain View, CA) was used. The TBLASTN algorithm (Altschul *et al.*, 1997) was used to search the databases at the National Center for Biotechnology Information (Bethesda, MD). The nucleotide sequence of *H. polymorpha* PYC1 (*HpPYC1*) was deposited at Genbank and was assigned Accession No. AF 221670.
Construction of mutants

A \textit{PYC1} disruption strain was constructed as follows: the \textit{H. polymorpha} \textit{URA3} gene (Merckelbach \textit{et al.}, 1993) was isolated as an \textit{Bg}II (blunt ended) - \textit{Pst}I fragment and ligated into pBKS-PYC ( \textit{Sty}I (blunt ended) - \textit{Pst}I fragment). From this plasmid (pBKS-\textit{ΔPYC::URA3}) a 3.1-kb \textit{Bg}II fragment was isolated and used to transform \textit{H. polymorpha} NCYC 495 (\textit{leu1.1 ura3}) or \textit{pex3::LEU2 (ura3 Δpex3}). Transformants were selected for uracil prototrophy and inability to grow on minimal methanol media. Correct integration was confirmed by Southern blotting.

A mutated \textit{HpPYC1} gene (R316Q), in which the codon encoding residue 316 (arginine) was substituted by glutamine, was constructed by overlap extension PCR (Horton \textit{et al.}, 1990) using primer PYC-1 (5’GACATTATTTCCGAAAATTAATCTCCTCAGATCCAGGGTCGAGCACACC3’) and both pBKS-40 universal (MF) and –50 reverse primers (MR). From the PCR product a 0.3-kb \textit{Pst}I/\textit{Nar}I fragment was exchanged with the same fragment in pBKS-PYC resulting in pBKS-PYCR316Q. For introduction into \textit{H. polymorpha} NCYC 495 (\textit{leu1.1 Δpyc1}) plasmid pYT3-PYCR316Q was constructed by ligation of the full-length mutated \textit{HpPYC1} gene (XbaI/EcoRI blunt ended) from the pBKS plasmid into pYT3 (XbaI/BamHI blunt ended) (Tan \textit{et al.}, 1995).

Plasmid pHIPX5 carrying \textit{HpPYC1} under control of AMO promoter (P\textsubscript{AMO}) was constructed as follows: \textit{PYC1}, amplified by PCR using primers “PYC-ATG” (CTTCCATGGCCCAGGTCG) and “PYC-STOP” (CCGCATGCAGAGCGAGACGC), was digested by \textit{NcoI} and \textit{SphI} and cloned into pHIPX5 digested with the same restriction enzymes. The resulting plasmid was linearized
with BsiWI and transformed into *H. polymorpha* NCYC 495 Δpyc1(leu1.1). A strain in which a single copy of the expression construct was integrated was selected, based on Southern blot analysis.

**Isolation of His$_6$-tagged HpPyc1p**

For isolation of C-terminal His$_6$-tagged HpPyc1p, *PYC1* was amplified by PCR, using primers “PYC-ATG” and “PYC-STOP”, digested with *NcoI/PvuII* and inserted into *NcoI/BglII* (blunt ended) digested pQE-60 (Qiagen GmbH, Hilden, Germany), resulting in plasmid pQE60-PYC. *E. coli* Sq13009[pREP4] containing pQE60-PYC was grown as detailed in *The QIAexpressionist*™. Cell pellets were suspended in a 50 mM potassium phosphate buffer pH 7.4, containing 300 mM NaCl; 0.2 mM β-ME, 10% glycerol, 0.2 mM EDTA and Complete™ (Roche). Cells were disrupted using a French Press. Cell debris and other insoluble material were removed by centrifugation. The cell extract was loaded onto a 1 ml Ni$^{2+}$ containing HiTrap™ Chelating column (Amersham Pharmacia Biotech AB, Sweden), washed with 10 ml buffer containing 75 mM imidazole and 150 mM NaCl in 50 mM potassium phosphate pH 7.4 and eluted with the same buffer containing 500 mM imidazole. The peak fractions contained highly purified HpPyc1p as determined by SDS-PAGE and Comassie brilliant blue staining (not shown).

**Biochemical methods**

Crude extracts were prepared according to van der Klei *et al.* (1991b). AO activity was measured as described by Verduyn *et al.* (1984). AO monomers and octamers were separated by sucrose density centrifugation (Goodman *et al.*, 1984). Protein
concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad GmbH, Munich, Germany) using bovine serum albumin as standard. The FAD content of AO was determined in immunoprecipitates by fluorescence spectroscopy as detailed previously (van der Klei et al., 1989a). SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and Western blotting (Kyhse-Andersen, 1984) was performed as described. Blots were decorated using antisera against various H. polymorpha proteins or Saccharomyces cerevisiae pyruvate carboxylase (Rohde et al., 1991) and the chromogenic or chemiluminescent Western Blotting kit (Boehringer Mannheim BV, Almere, the Netherlands). Cell fractionation was performed as described by van der Klei et al. (1998). A post-nuclear supernatant (10 ml in total) was loaded onto a discontinuous sucrose gradient (25 ml). After centrifugation 1.5 ml fractions were taken from the bottom.

**AO/HpPyc1p binding studies.**

AO and BSA columns were prepared as described by Evers et al. (1993). Upon binding the proteins were denatured by incubation for 16 hours at 4 °C in a buffer containing 8M urea in 25 mM Tris-HCl pH 7.0 followed by extensive washing with buffer A (25 mM Tris HCl, 50 mM KCl, 1 mM DTT, pH 7.0) to remove the urea. For binding studies 100 µl purified HpPyc1p (50 µg/ml) was loaded onto 50 µl columns, followed by washing with 20 column volumes buffer A and elution with 20 column volumes of a solution containing 8M urea. Proteins were precipitated with TCA and analyzed by Western blotting.
**Fluorescence correlation spectroscopy (FCS)**

The FCS setup was basically as described by Hink and Visser (1998). For excitation of FAD, argon ion laser lines of 488 nm were used. The light intensity was adjusted by using various neutral density filters. Measurements were made in a 96-well chamber in 50 mM potassium phosphate buffer pH 7.0 at room temperature. For the analysis of FAD binding to HpPyc1p, purified HpPyc1p (200 or 400 nM) was incubated with FAD (100 nM) for 1 h at 37° C prior to the measurements. The concentrations of FAD and pyruvate carboxylase were calculated based on their extinction coefficients (FAD $\varepsilon_{450} = 11.3 \text{ mM}^{-1} \text{ cm}^{-1}$, HpPyc1p monomer $\varepsilon_{280} = 77 \text{ mM}^{-1} \text{ cm}^{-1}$). Diffusion constants of individual fluorescent molecules were calculated from the time dependent fluctuation of the fluorescent signal. Experimental autocorrelation curves were then fitted by theoretical autocorrelation functions using the FCS Data Processor 1.3 software. In all series of experiments the alignment and focussing of the setup was frequently checked by measuring the autocorrelation function of 7.6 nM rhodamin 110. The dimensions of the excitation volume were determined by the known diffusion coefficient of rhodamin 110.

**Electron microscopy**

Whole cells were fixed and prepared for electron microscopy as described (Waterham *et al.*, 1994). Immunolabeling was performed on ultrathin sections of Unicryl-embedded cells, using specific polyclonal antibodies against various *H. polymorpha* and *S. cerevisiae* proteins and gold-conjugated goat-anti-rabbit (GAR) antibodies (Waterham *et al.*, 1994). Cytochemical staining experiments for the detection and localization of AO activity were performed by the CeCl3-based method (Veenhuis *et al.*, 1976).
Results.

The *Hansenula polymorpha* pyruvate carboxylase gene functionally complements a mutant defective in alcohol oxidase assembly.

In a genetic approach to identify proteins involved AO assembly/activation, we have isolated a collection of *H. polymorpha* mutants that are impaired to utilize methanol as sole carbon source (Mut⁻ phenotype) due to strongly reduced or absent AO activities (Van Dijk *et al.*, 2002). Four mutants were characterized by normal AO protein levels, but strongly reduced AO enzyme activities. Localization studies revealed that the import of AO into peroxisomes was specifically blocked in these mutants (Van Dijk *et al.*, 2002). The overall morphology of methanol-induced cells of a representative strain of these mutants, *ass3*-110, compared to WT cells, is shown in Fig. 1. Growth experiments revealed that these 4 mutants also displayed, in addition to the Mut⁻ phenotype, a severe growth defect on minimal glucose-ammonium sulphate media, which could be restored by addition of aspartate or glutamate. Addition of these amino acids, however, did not result in the complementation of the Mut⁻ phenotype. The amino acid requirement (Asp⁻/Glu⁻) could not be separated from the Mut⁻ phenotype through backcrosses with parental strains indicating that both phenotypes were closely linked. Complementation analysis revealed that all 4 mutants with this phenotype (Mut⁻, Asp⁻/Glu⁻) fell in one complementation group, designated *ass3*.

To isolate the defective gene, strain *ass3*-110 was transformed with a genomic *H. polymorpha* library. Transformants capable to grow on mineral media containing methanol (Mut⁺, Asp⁺/Glu⁺) were selected. Subcloning and re-introduction of the complementing fragments into *ass3*-110 resulted in a 4.2 kb genomic fragment that
contained the complementing activity. This fragment was sequenced and the sequence was deposited at GenBank (Accession No AF 221670).

Sequence analysis revealed that the complementing fragment contained a potential open reading frame (ORF) encoding a protein of 1175 amino acids with a predicted MW of 130 kDa. A database search revealed that this protein was highly similar throughout the entire protein to pyruvate carboxylases (Pyc) from various organisms ranging from bacteria (*e.g.* *Bacillus subtilis* 50% identity) and yeast (*Pichia pastoris*, 81% identity; *Saccharomyces cerevisiae* Pyc1p and Pyc2p, both 77% identity, to man (53% identity). Based on this we designated the gene *PYC1* and its translation product HpPyc1p.

An *H. polymorpha* *PYC1* disruption mutant (*∆pyc1*) was constructed in which approximately half of the *H. polymorpha* *PYC1* gene was deleted (the region encoding amino acids 298-905). Growth experiments indicated that cells of *∆pyc1* showed the same phenotype as the original mutant *ass3-110*: no growth on minimal media containing glucose and ammonium sulphate unless aspartate or glutamate were added and a defect in growth on methanol independent of the presence of aspartate or glutamate.

Mating of the *∆pyc1* strain with the original *ass3-110* mutant resulted in diploids that were all Mut-. After sporulation, no Mut*+* cells were observed, demonstrating that *ass3-110* and *∆pyc1* are closely linked and most likely are alleles of the same gene.

Pyc is an anapleurotic enzyme that replenishes the tricarboxylic acid (TCA) cycle with oxaloacetate from pyruvate. For *S. cerevisiae* it has been shown that the absence of Pyc results in the inability of cells to grow on minimal media containing glucose and ammonium sulphate, while they do grow on glucose-aspartate containing media (Stucka *et al.*, 1991). Also *P. pastoris* mutants lacking Pyc were reported to be unable to grow on
glucose-ammonium sulphate media, whereas growth is possible with aspartate or glutamate as nitrogen source.

These data indicate that the Asp'/Glu' phenotype of *H. polymorpha Δpyc1* is due to the absence of Pyc enzyme activity. However, it does not explain why *H. polymorpha Δpyc1* cells are unable to grow on methanol media that contain Asp or Glu.

**Properties of AO in *H. polymorpha Δpyc1* cells**

AO enzyme activity measurements in crude extracts prepared from methanol-induced Δpyc1 and WT control cells, revealed that the AO activity of Δpyc1 cells was less than 2% of the activity found in WT cells (0.07 and 4.1 U/mg protein, respectively). Western blot analysis of these extracts revealed, however, that the AO protein levels were normal in Δpyc1 cells (Fig. 2). Also, the amounts of other peroxisomal matrix enzymes (dihydroxyacetone synthase [DHAS] and catalase [CAT]) and the peroxins Pex3p, Pex5p and Pex14p were virtually identical in WT and Δpyc1 cells (Fig. 2).

To analyze whether the absence of AO activity was due to a defect in AO oligomerization, crude extracts were subjected to sucrose density gradient centrifugation in order to separate AO monomers from octamers (Goodman *et al.*, 1984). Western blot analysis revealed that in gradients prepared from WT control cells almost all AO protein was found in the bottom fractions (Fig. 3, fraction 6) where octameric AO sediments. However, in gradients prepared from methanol-induced Δpyc1 cells AO protein was found in the top fractions indicative for a monomeric state (Fig. 3, fraction 2 and 3). In the fractions where octameric AO sediments no AO protein was detected. However, because the cells still display some enzyme activity (less than 2% of the enzyme activity in WT
cells) and only octameric AO is active, the octameric AO apparently is below the level of detection in the experiment shown in Fig. 3.

Fluorescence analysis of the FAD content of AO protein, immunoprecipitated from crude extracts of WT and Δpyc1 cells, revealed that in precipitates of equal amounts of AO protein from crude extracts prepared from Δpyc1 or WT cells the concentration of FAD was approximately 25-fold lower in precipitates from Δpyc1 cells compared to WT controls.

The overall morphology of methanol-induced H. polymorpha Δpyc1 cells was highly comparable to that of the original ass3-110 cells. Immunocytochemically, anti-AO specific labelling was predominantly localized in the cytosol, with very little labelling on peroxisomes (Fig. 4A). However, the other major PTS1 proteins DHAS (Fig. 4B) and CAT (Fig. 4C) showed a normal peroxisomal location. Cytochemical staining experiments revealed that in Δpyc1 AO enzyme activity was, like in WT cells, confined to peroxisomes (Fig. 4D), although the amount of activity fluctuated between individual organelles, reflected by variations in staining intensity. Although the level of AO activity is very low in Δpyc1 cells, this can easily be detected by cytochemical staining, because this technique is extremely sensitive.

Taken together, these data indicate that in H. polymorpha Δpyc1 cells bulk of the AO protein is in the cytosol in an inactive, FAD-lacking, monomeric form while a minor fraction is present as enzymatically active, FAD-containing octamers inside peroxisomes.

The AO assembly failure in Δpyc1 cells is not indirect and due to an import defect.
The failure of AO assembly in Δpyc1 cells may be related to a spatial separation of the AO monomers (in the cytosol) and putative peroxisomal assembly factor(s). In H. polymorpha pex mutants these peroxisomal factor(s) most likely are also mislocalized to the cytosol, thus explaining why in these cells AO assembly/activation normally occurs in this compartment (van der Klei et al., 1991c). In Δpyc1 cells, however, normal peroxisomes are still present that may contain the putative AO assembly factor(s), as a peroxisomal protein import defect other than for AO protein was not observed. To test this possibility, we constructed a H. polymorpha Δpex3Δpyc1 double mutant, in which all peroxisomal matrix proteins are predicted to be mislocalized to the cytosol (Baerends et al., 1996). Biochemical analysis of crude extracts of methanol-induced Δpex3Δpyc1 cells showed that the level of various peroxisomal enzymes and peroxins was normal (Fig. 2). However, AO assembly was not restored, because very low specific AO activities were detected (data not shown). Also, sucrose density gradient analysis of crude extracts prepared from methanol-induced Δpex3Δpyc1 cells revealed that, like in Δpyc1, AO was predominantly present in a monomeric state (Fig. 3). Controls, prepared from crude extracts of Δpex3 cells, confirmed that the absence of peroxisomes in these cells did not influence AO oligomerization, as bulk of the AO protein was found in fractions where octameric AO sediments (fraction 6,7; Fig. 3). These results suggest that the AO assembly defect in Δpyc1 cells is not an indirect effect, due to a specific AO protein import block.

**AO assembly does not require HpPyc1p enzyme activity**
To test whether AO assembly is dependent on HpPyc1p enzyme activity, we introduced a point mutation in *H. polymorpha PYC1* that replaces the active site residue arginine 316 by glutamine (R316Q). Indeed, this mutant was unable to grow on minimal medium containing glucose unless supplemented with aspartate or glutamate (data not shown). However, in the presence of these amino acids, cells of the mutant strain could grow on methanol, indicative for the restoration of the AO assembly defect (Fig. 5A). Western blotting experiments using antibodies against *S. cerevisiae* Pyc protein, which cross react with HpPyc1p (see Fig. 2), revealed that HpPyc1p\textsuperscript{R316Q} was synthesized in methanol-induced cells to levels comparable to WT cells (Fig. 5B). Immunocytochemical analysis confirmed that AO protein was exclusively present in peroxisomes of these cells, indistinguishable from WT cells (Fig. 5C). Hence, not the enzyme activity but another function of the protein is required for AO assembly.

**HpPyc1p is a cytosolic enzyme**

In order to determine the subcellular location of HpPyc1p, homogenized protoplasts prepared from methanol-grown WT cells were subjected to sucrose density centrifugation followed by Western blot analysis of the various fractions obtained. As shown in Fig. 6, HpPyc1p was only detected in the low density fractions at the top of the gradient (Fig. 6, fractions 18-24), indicative for a cytosolic location. Analysis of the peroxisomal peak fractions did not reveal any HpPyc1p, indicating that the protein does not partially co-sediment with peroxisomes. A cytosolic location is in line with the reported location of Pyc protein in *S. cerevisiae* (Walker et al., 1991).
Newly induced HpPyc1p can mediate assembly of cytosolically accumulated AO monomers

The intriguing question concerns the function of HpPyc1p in AO assembly: does it act like a chaperone or does it serve other functions, e.g. in co-factor binding? This question was addressed in a Δpyc1 strain that contained a copy of the PYC1 gene under control of the inducible amine oxidase promoter (P_{AMO}). In this strain, Δpyc1::P_{AMO}PYC1, the synthesis of AO monomers and the HpPyc1p protein can be separated in time. First, cells of this strain were induced on methanol/ammonium sulphate, conditions that induce the synthesis of AO monomers, but largely represses HpPyc1p synthesis due to the presence of ammonium sulphate. Subsequently, the cells were shifted to glucose/ethylamine, conditions that strongly repress AO synthesis (due to the presence of glucose) but induce HpPyc1p synthesis due to the induction of the P_{AMO} by ethylamine. This allowed addressing the question whether existing, monomeric AO molecules that had accumulated in the cytosol, were still accessible for the HpPyc1p function in AO assembly. The results depicted in Fig. 7 show that HpPyc1p was below the limit of detection in Δpyc1::P_{AMO}PYC1 cells prior to the shift (Fig. 7A, lane 4 T = 0 min). After the shift to ethylamine containing media PYC1 was rapidly induced and HpPyc1p levels comparable to WT were detected within 60 min. (compare lane 1-3 with lane 6). Western blot analysis of a native gel confirmed that significant amounts of monomeric AO had accumulated prior to the shift to ethylamine as nitrogen source. However, within 30 min. after the induction of HpPyc1p synthesis, the monomeric AO band had disappeared. At the same time the level of AO enzyme activity (Fig. 7C) had significantly increased. In a control experiment using WT cells, the level of AO activity and octameric AO decreased,
as a result of glucose-induced degradation of peroxisomes. This process most likely also occurs in Δpyc1::P_{AMO}PYC1 cells, as indicated by the subsequent reduction in AO protein and activity levels that follow the initial strong increase (Fig. 7C).

**HpPyc1p physically interacts with AO and FAD**

To study whether HpPyc1p has affinity for AO protein, *in vitro* binding studies were performed. To this purpose a Sepharose column containing alcohol oxidase protein was prepared (Evers *et al.*, 1993). As a control, immobilized bovine serum albumine (BSA) was used. Purified HpPyc1p was loaded onto these columns. Upon extensive washing, the bound protein was eluted by a buffer containing 8M urea. As shown in Fig. 8, a significant portion of the loaded HpPyc1p protein had bound to the AO column, whereas in the control experiment using BSA all HpPyc1p was found in the flow through fraction. These findings indicate that HpPyc1p is capable to bind to AO protein.

In order to study whether HpPyc1p could play a role in the association of FAD to AO, we tested whether HpPyc1p, which contains an ATP binding motif, is capable of binding FAD. FAD binding was measured using Fluorescence Correlation Spectroscopy (FCS), a technique that allows to measure diffusion constants of fluorophores. Analysis of normalized fluorescence autocorrelation curves of FAD in the absence of HpPyc1p revealed that the average diffusion time of FAD, obtained by a one-component fit analysis, was 41.53 µs. This value is in agreement with the molecular weight of FAD molecules. After addition of purified HpPyc1p, the fluorescence autocorrelation curve changed and could be best fitted with a two-component fit. Fixing the average diffusion time of FAD, the diffusion time of the second component was 191-213 µs. Using the
equation $\text{MW}_{\text{PYC}} = (\tau_{\text{PYC}}/\tau_{\text{FAD}})^3 \times \text{MW}_{\text{FAD}}$ the molecular weight of the second component was calculated to be 105 - 130 kD. Assuming the HpPyc1p molecules to be spherical in shape, this is in agreement with the apparent molecular weight of monomeric HpPyc1p calculated from its amino acid sequence (130 kDa). Hence, these data indicate that part of the FAD had bound to the added HpPyc1p. In control experiments using lysozyme instead of HpPyc1p no change of fluorescence autocorrelation curve and diffusion time were observed.

**Also in Pichia pastoris Pyc1p is essential for AO import and assembly.**

In order to determine whether the Pyc1p-dependent AO assembly defect is limited to *H. polymorpha* or represents a common feature of methylotrophic yeast, we analyzed a *PYC* deletion strain of *Pichia pastoris* (Menendez et al., 1998). The *P. pastoris* Δpyc strain, like its *H. polymorpha* counterpart, was unable to grow on glucose unless aspartate or glutamate was added. Growth on methanol was fully prevented, irrespective of the presence of these amino acids in the media. Immunocytochemical experiments revealed that, like in *H. polymorpha*, *P. pastoris* Δpyc cells did not import AO in peroxisomes (Fig. 9B) as in WT cells (Fig. 9A).

Moreover, a *P. pastoris pyc1* suppressor mutant (Menendez et al., 1998), in which the aspartate requirement was restored, still failed to assemble AO and thus, to grow on methanol (data not shown), indicating that also in *P. pastoris* Pyc protein, but not the enzyme activity, is required for AO assembly.

Remarkably, *P. pastoris* Δpyc cells grew normally on oleic acid, at rates similar to WT controls. Hence, the absence of Pyc1p has no general deteriorating effect on peroxisome
biogenesis or function. Also, one of the key enzymes of oleate metabolism is a peroxisomal flavoprotein, namely acyl-CoA oxidase. This protein displays normally activities (not shown) and is located in peroxisomes (Fig. 9C). Therefore, the PYC1 deletion seems to interfere specifically with AO assembly and not with that of other peroxisomal flavin proteins.
Discussion.

We have identified pyruvate carboxylase (Pyc) as the first protein that has an essential function in assembly of peroxisomal alcohol oxidase (AO) in methylotrophic yeast. Pyc is an anapleuroetic enzyme that replenishes the tricarboxylic acid cycle with oxaloacetate from pyruvate. As a consequence yeasts lacking Pyc enzyme activity cannot grow on minimal glucose media unless aspartate or glutamate is added, amino acids that can be converted into oxaloacetate. Unexpectedly, *Hansenula polymorpha* and *Pichia pastoris* strains lacking Pyc are also unable to grow on methanol, independent of the presence of aspartate or glutamate in the medium. We demonstrated that this growth defect is due to a severe block in the assembly of alcohol oxidase (AO), a key enzyme in methanol metabolism. Our data convincingly show that Pyc protein, but not its enzyme activity, is necessary for AO assembly. The import and activation of another peroxisomal flavin enzyme, acyl-CoA oxidase, was not affected in the absence of Pyc. Hence, Pyc seems to function specifically in the AO assembly pathway in methylotrophic yeast.

The current model of AO assembly hypothesizes that AO monomers, but not octamers, are imported into peroxisomes. This is based on the finding that octameric AO protein is not incorporated into peroxisomes *in vivo* (Douma *et al*., 1990; Waterham *et al*., 1993) and on the results of elegant pulse chase experiments that provided evidence that assembly into the active octamer takes place inside the organelle (Goodman *et al*., 1984; Stewart *et al*., 2001).

FAD most likely binds to monomeric AO, as is indicated by the finding that FAD can not re-associate *in vivo* to AO octamers, from which FAD has been chemically removed (van
der Klei et al., 1989a). Whether in \textit{H. polymorpha} FAD binds to AO monomers prior to or upon translocation across the peroxisomal membrane was so far still speculative. We show here that in \textit{H. polymorpha Δpyc1} cells bulk of the AO protein accumulated as inactive, FAD-lacking monomers in the cytosol. Only a minor portion had assembled into FAD-containing, enzymatically active octamers, which - based on cytochemical experiments - were localized in the peroxisomal matrix. This suggests that the presence of HpPyc1p is important for FAD-binding to AO monomers, the subsequent translocation into the peroxisomal matrix and finally the assembly into octamers.

Interestingly, the phenotype of \textit{H. polymorpha Δpyc1} cells is highly comparable to that of the \textit{H. polymorpha} riboflavin auxotrophic mutant, \textit{rif1} (Evers et al., 1996), in which also bulk of the AO protein accumulates as FAD-lacking monomers in the cytosol. A likely way to explain both observations is that FAD binding to AO monomers in the cytosol is a prerequisite to allow efficient translocation into peroxisomes. Our current findings suggest that cytosolically located HpPyc1p is required to bind FAD to AO monomers.

We found that in HpPyc1p-deficient cells soluble, monomeric AO accumulated that could be activated upon subsequent artificial induction of \textit{PYC1} expression. Together with the observation that HpPyc1p physically interacts with AO protein and is capable to bind FAD, our current data strongly suggest that HpPyc1p functions as a FAD-binding protein in the cytosol.

Relatively little is known on proteins that play a role in co-factor binding. It is generally assumed that co-factor binding occurs spontaneously upon formation of the correct binding site in a protein molecule. So far no proteins have been described that play a role in non-covalent binding of FAD; also, only one is yet identified that is essential for
covalent FAD binding (Kim et al., 1995). Several examples are known of proteins involved in binding of heme, for instance mitochondrial heme lyases that are necessary for the attachment of heme to cytochrome c or cytochrome c1 (Page et al., 1998). However, despite extensive research molecular details on their mode of action are still lacking.

An alternative explanation for the phenotype of H. polymorpha Δpyc1 is that HpPyc1p is essential to mediate binding of AO to the receptor Pex5p and that FAD-binding occurs after import mediated by a peroxisomal factor. However, this explanation is less likely in view of the fact that in cells of the Δpex3Δpyc1 double mutant AO remains monomeric, while it is normally active in single pex mutants, e.g. Δpex3 (compare Fig. 3) and Δpex5 (van der Klei et al., 1995).

We showed before, that FAD-containing AO monomers can assemble spontaneously into octamers in vitro (Evers et al., 1995). Hence, it can be envisaged that this also can take place in intact cells in vivo. However, when FAD-binding indeed occurs in the cytosol the cell has to deal with the problem how to prevent premature spontaneous assembly of the FAD-containing monomers. There is a strong metabolic need for the cell to postpone this event until import has occurred, since only minor amounts of active AO in the cytosol give rise to severe energetical disadvantages due to a cytosolic H2O2 metabolism that would retard or even prevent growth on methanol (van der Klei et al., 1991b). One possibility is that HpPyc1p remains bound to AO after co-factor binding. A second option is that, upon FAD binding, the protein is immediately donated to Pex5p, which prevents octamer formation. Since other PTS1 proteins (DHAS, CAT) are normally imported in Δpyc1 cells and also their Pex5p levels are similar to WT cells, it is indeed unlikely that
Pex5p molecules are bound to the large pool of FAD-lacking AO monomers in the cytosol of \( \Delta pyc1 \) cells.

Based on the above reasoning, our adapted hypothetical model of AO assembly predicts that the first step in AO import is HpPyc1p-mediated FAD binding to newly synthesized AO protein in the cytosol (Fig. 10). Subsequently, the FAD-containing monomers bind to the PTS1 receptor Pex5p, followed by the translocation of the Pex5p-AO cargo complex into peroxisomes. After dissociation of the cargo from Pex5p in the organellar matrix, the FAD-containing AO monomers may spontaneously oligomerize into enzymatically active octamers followed by shuttling of Pex5p back to the cytosol to mediate another round of PTS1 protein import. This model is in line with the previously proposed "extended shuttle model" for Pex5p (van der Klei et al., 1995; van der Klei and Veenhuis, 1996), which was recently experimentally proven by Dammai and Subramani, (2001) for human cells.

A further implication of the present study is that in methylotrophic yeasts Pyc has multiple functions. The relevance of this is obvious in view of the data on the human genome that have become available recently (Lander et al., 2001; Venter et al., 2001). The 30,000 genes that have been identified cannot cope for the multitude of functions that are predicted to be essential in men unless specific genes encode multiple proteins or proteins with multiple functions. Proteins that fulfil two different functions are the housekeeping enzyme lactate dehydrogenase B in duck which is also the lens structural protein epsilon-crystallin (Hendriks et al., 1988), glyceraldehyde 3-phosphate dehydrogenase that has been shown to play a role in endocytosis in CHO cells (Robbins et al., 1995) and the \( \alpha \)-subunit of phosphofructokinase (but not enzyme activity) that is
required for the onset of glucose-mediated selective pexophagy (microautophagy; Yuan et al., 1997). The switch in function may even be temperature-dependent, as demonstrated for heat shock protein DegP that normally functions as a chaperone, but displays proteolytic activity at elevated temperatures (Spiess et al., 1999).

Our collection of Ass mutants that comprises 10 different complementation groups (Van Dijk et al., 2002), suggests that additional proteins may be involved in AO biosynthesis (e.g. targeting or translocation). This view is also based on the finding that in P. pastoris the carboxyterminal PTS1 signal is not the only targeting information essential for import (Waterham et al., 1997). Therefore specific additional proteins may exist that exclusively function in AO import and assembly. We are currently trying to identify these proteins by cloning the encoding genes by functional complementation of the other ass mutants.
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**Abbreviation list**

Abbreviations: AO, alcohol oxidase; BSA, bovine serum albumine, CAT, catalase; DHAS, dihydroxyacetone synthase; FAD, flavin adenine dinucleotide; FCS, fluorescence correlation spectroscopy.
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Figure legends

Figure 1. Morphology and immunocytochemistry of methanol-induced cells of WT *H. polymorpha* (Fig. 1A) and *ass3-110* cells (Fig. 1B,C). WT cells (Fig. 1A) are characterized by the presence of several large peroxisomes that harbor AO protein (*α*-AO antibodies). Only small peroxisomes are found in cells of the mutant strain (Fig. 1B; KMnO₄-fixation). Electron micrographs are taken of glutaraldehyde-fixed cells, poststained with uranylacetate unless otherwise indicated. Abbreviations: M - mitochondrion; N - nucleus; P - peroxisome, V - vacuole, * - AO protein aggregate. The marker represents 0.5µm.

Figure 2. Western blot analysis of crude extracts prepared from methanol-induced *H. polymorpha* WT, Δpyc1 and Δpex3Δpyc1 cells. The blots were decorated with antibodies against various *H. polymorpha* proteins. HpPyc1p was detected using antibodies against *S. cerevisiae* Pycp that crossreact with the *H. polymorpha* protein. The proteins are present in virtually equal amounts except that Pex5p levels are slightly enhanced in Δpex3Δpyc1 cells. Equal amounts of protein were loaded per lane.

Figure 3. Analysis of the oligomerization state of AO protein in *H. polymorpha* WT, Δpyc1, Δpex3Δpyc1 and Δpex3 cells by sucrose density gradient centrifugation of crude extracts prepared from methanol-induced cells. All fractions of the gradient were analyzed for the presence of AO protein by Western blotting. Fraction 1 represents the top fraction, fraction 8 the bottom fraction. Monomeric AO sediments to fractions 2-3, octameric AO to fractions 6-7. Equal portions of the fractions were loaded per lane.
Figure 4. Immunocytochemical demonstration of peroxisomal matrix proteins in methanol-induced *H. polymorpha Δpyc1* cells. Using anti-AO antisera labeling was predominantly observed in the cytosol, whereas only a minor portion of the protein was localized at peroxisomes (A). Characteristically, the labeling intensities of the peroxisomal profiles strongly varied. Specific anti-DHAS (B) or anti-catalase (C) dependent labeling was confined to peroxisomes. Cytochemical staining for the detection of AO enzyme activity (D) revealed that the enzyme activity was invariably confined to peroxisomes. Like AO protein, also the staining intensity varied (indicated by arrows) among the peroxisomal population, present in one cells, indicative for variations in the levels of active AO protein.

Figure 5. Enzymatically active HpPyc1p is not required for AO assembly.

\(A\) Growth of *H. polymorpha* WT (●), *H. polymorpha Δpyc1* producing the mutant protein HpPyc1p\(^{R316Q}\) (▼) and *H. polymorpha Δpyc1* containing an empty expression plasmid (○) on mineral media containing methanol supplemented Asp and Glu. *H. polymorpha Δpyc1* cells are severely hampered in growth (OD\(_{660}\) = 0.6 after 36 h). The initial increase in cell density is due to the presence of small amounts of yeast extract in the media. In contrast, cells of *H. polymorpha* WT and the Δpyc1 strain producing HpPyc1p\(^{R316Q}\) are able to grow on methanol and reached a comparable final yield (OD\(_{660}\) 3.2 and 2.5 after 36 h. of growth, respectively; the cell density is expressed as optical density at 660 nm; OD\(_{660}\)).
(B) Comparison of the levels of HpPyc1p in crude extracts prepared from methanol-induced *H. polymorpha* strains using Western blotting and antibodies against *S. cerevisiae* Pycp. Lane 1 WT; lane 2-5 Δpyc1 cells containing no plasmid (2), an empty expression plasmid (3), a plasmid containing WT *HpPYC1* (4) or a plasmid containing mutant *PYC1-R316Q* (5). Equal amounts of protein were loaded per lane.

(C) Immunocytochemical demonstration of AO protein in methanol-induced Δpyc1 cells producing HpPyc1p containing the mutation R316Q. Anti-AO specific labeling was confined to peroxisomes.

Figure 6. Sucrose gradient, prepared from a post-nuclear supernatant obtained from methanol-grown *H. polymorpha* WT cells. The graph shows the distribution of the peroxisomal marker enzyme AO (+), the mitochondrial marker enzyme cytochrome c oxidase (▼), the protein (0) and sucrose concentrations (.....). The Western blot shows the distribution of HpPyc1p in the even fractions of the gradient. The protein was only detected in the upper part of the gradient (fractions 18-24), which corresponds to the cytosol. Sucrose concentrations are expressed as % (w/w), the protein concentrations as mg/ml and the specific activities of AO and cytochrome c oxidase as percentages of the value in the peak fractions that were arbitrarily set at 100.

Figure 7. Biochemical demonstration of the assembly of cytosolically accumulated monomeric AO upon subsequent artificial induction of *PYC1*. Cells of the Δpyc1::PAmoPYC1 strain were pregrown on methanol/ammonium sulphate to induce AO synthesis under conditions that *PYC1* expression is strongly repressed by ammonium
sulphate. Subsequently the cells were shifted (at $T = 0$ min.) to media containing glucose and ethylamine to induce $P_{AMO}$, and thus HpPyc1p production and to repress AO synthesis. Fig. 7A represents a Western blot of an SDS-PAA gel prepared from crude extracts of WT and $\Delta pyc1::P_{AMO}PYC1$ cells taken prior to ($T = 0$ min.) or 30 and 60 min. after the shift. The blot, decorated with anti-Pyc antibodies, shows the induction profile of HpPyc1p in $\Delta pyc1::P_{AMO}PYC1$ cells, compared to WT levels. In Fig. 7B a Western blot of a native gel of the same samples is shown. This blot, decorated with anti-AO antibodies, which allows to detect both octameric AO (O) or monomeric AO (M), shows that monomeric AO present at $T = 0$ min. is undetectable at $T = 30$ min. Fig. 7C represents the enzyme activities of AO in the same samples (black bar: WT cells; gray bar: $\Delta pyc1::P_{AMO}PYC1$ cells). The reduction in AO activities in WT cells is due to selective peroxisome degradation, induced by glucose. The values in both strains at $T = 0$ min. were set to 100% (absolute values at T=0h for WT 2.0 U/mg protein and $\Delta pyc1::P_{AMO}PYC1$ cells 0.5 U/mg protein).

Figure 8. *In vitro* interaction of AO and HpPyc1p. Purified HpPyc1p was loaded onto columns containing AO or BSA protein. The columns were subsequently extensively washed. The flow through and wash fractions were pooled (F). Bound HpPyc1p was eluted using a buffer containing 8M urea (E). Equal portions of F and E were subjected to SDS-PAGE and blotted. The blots were decorated using anti-Pyc antibodies. Most HpPyc1p was found in the elution fraction (E) when AO columns were used, in contrast all HpPyc1p was found in the flow through and wash fraction (F) when a control column containing BSA was used.
Figure 9. Immunocytochemical localization of AO (A, B) and acyl-CoA oxidase (C) in *P. pastoris* WT (A) and ∆pyc cells (B, C). In methanol-grown WT cells anti-AO dependent labelling is confined to peroxisomes (A), whereas in ∆pyc cells (B) the protein was predominantly detected in a cytosolic aggregate (*) but not in peroxisomes. In oleic acid-grown ∆pyc cells the anti-acyl-CoA oxidase dependent labeling is confined to peroxisomal profiles (C).

Figure 10. Hypothetical model of AO import and assembly in *Hansenula polymorpha*. AO monomers are synthesized on free ribosomes in the cytosol. In the cytosol HpPyc1p assists in binding of the co-factor FAD to newly synthesized AO monomers. Subsequently, the FAD-containing monomers are bound to the PTS1 receptor, Pex5p. Both the receptor and its cargo are translocated across the peroxisomal membrane (I), followed by dissociation of the AO-Pex5p complex in the organellar matrix. The FAD-containing AO monomers then assemble into octamers. Pex5p recycles to the cytosol (II) to mediate another round of PTS1 protein import.