The methylotrophic yeast *Hansenula polymorpha* contains an inducible import pathway for peroxisomal matrix proteins with an N-terminal targeting signal (PTS2 proteins)

(peroxisomes/protein import/amine oxidase/malate dehydrogenase/thiase)

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**ABSTRACT** Two main types of peroxisomal targeting signals have been identified that reside either at the extreme C-terminus (PTS1) or the N-terminus (PTS2) of the protein. In the methylotrophic yeast *Hansenula polymorpha* the majority of peroxisomal matrix proteins are of the PTS1 type. Thus far, for *H. polymorpha* only amine oxidase (AMO) has been shown to contain a PTS2 type signal. In the present study we expressed *H. polymorpha* AMO under control of the strong endogenous alcohol oxidase promoter. Partial import of AMO into peroxisomes was observed in cells grown in methanol/(NH₄)₂SO₄-containing medium. However, complete import of AMO occurred if the cells were grown under conditions that induce expression of the endogenous AMO gene. Similar results were obtained when the heterologous PTS2 proteins, glyoxysomal malate dehydrogenase from watermelon and thiase from *Saccharomyces cerevisiae*, were synthesized in *H. polymorpha*. The import of PTS1 proteins, however, was not affected by the growth conditions. These results indicate that the reduced rate of AMO import in (NH₄)₂SO₄-grown cells is not due to competition with PTS1 proteins for the same import pathway. Apparently, AMO is imported via a separate pathway that is induced by amines for functions for PTS2 proteins in general.

In the methylotrophic yeast *Hansenula polymorpha* the development of peroxisomes is induced by a number of unusual carbon and nitrogen sources (1, 2). In particular, during growth on methanol many large peroxisomes develop. These organelles contain the key enzymes alcohol oxidase (AO), dihydroxyacetone synthase (DHAS), and catalase (CAT) of methanol metabolism. Less pronounced induction of the organelles is observed during growth on various organic nitrogen sources—e.g., primary amines, d-amino acids, and purines.

Two different types of peroxisomal targeting signals (PTSs) have been identified that are essential and sufficient to direct newly synthesized precursor proteins to their target organelle. The first PTS identified was the tripeptide SKL, located at the extreme C-terminus of firefly luciferase (3, 4). Subsequently, this motive, and degenerate forms of it, were shown to be functional in mammals, insects, plants, and yeasts as well (4, 5). The second PTS type was found in rat thiase and comprises the first 11 amino acids of the thiase presequence (6, 7). Comparisons to other microbody proteins revealed a similar sequence in peroxisomal thiases from yeast (*Saccharomyces cerevisiae*, *Candida tropicalis*, and *Yarrowia lipolytica*) as well as other matrix proteins that lack a clear PTS1—namely, amine oxidase (AMO) from *H. polymorpha*, malate dehydrogenase (MDH) from watermelon, and aldolase from *Trypanosoma brucei*. On the basis of the sequence identities, a general consensus, RL-X₅-H/QL, has been proposed (5). Deletion analysis and site-directed mutagenesis confirmed the presence of an N-terminal targeting signal in *S. cerevisiae* thiase and watermelon MDH (8, 9). However, also substitutions outside the consensus appear to affect targeting of the protein (8, 10).

PTS1 and PTS2 proteins not only contain different targeting signals but also may use separate import pathways (11). This was concluded from the isolation of various peroxisome-deficient yeast strains (PER3/PAS8/PAS10) that are blocked in the import of PTS1 proteins only, while PTS2 proteins—e.g., thiase—are correctly sorted to peroxisomes (refs. 12–14; M.V., unpublished results). Moreover, a *S. cerevisiae* mutant has been isolated with the opposite phenotype—namely, proper import of PTS1 proteins and cytosolic location of thiase (14, 15).

In *H. polymorpha*, most of the peroxisomal matrix proteins identified to date contain a PTS1 signal—e.g., AO, DHAS, and CAT (16, 17). However, we have obtained strong indications that peroxisomal AMO, a key enzyme in amine metabolism, contains an N-terminal, PTS2-like signal (RL-X₅-QA). Deletion analysis revealed that essential targeting information is located within the the extreme N-terminal 16 amino acids. Moreover, this sequence can direct a reporter protein, bacterial β-lactamase, to the peroxisomal matrix of *H. polymorpha* (K.N.F., W.H., G.AB, and M.V., unpublished results). To obtain more information on the PTS2 import machinery we have expressed AMO under the control of the endogenous AO promoter (P_AO), leading to unusually high levels of this protein under methylotrophic growth conditions. In this paper we present evidence that efficient import of the “heterologous” AMO protein as well as other heterologous PTS2 proteins is dependent on induction of a specific component(s) of the import machinery.

**MATERIALS AND METHODS**

**Yeast Strains and Growth Conditions.** Transformants of *H. polymorpha* A16 (18), carrying the AMO gene under the control of the P_AO (H. polymorpha) GF15 and GF16; see below), were grown in C source-limited chemostat cultures on mineral medium containing a mixture of glucose (0.25%).

Abbreviations: AO, alcohol oxidase; DHAS, dihydroxyacetone synthase; CAT, catalase; AMO, amine oxidase; PTS, peroxisomal targeting signal; MDH, malate dehydrogenase; gMDH, glyoxysomal MDH; WT, wild type; D, dilution rate.

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wt/vol) and methanol (0.25%, vol/vol) as carbon source and (NH₄)₂SO₄ (0.25%, wt/vol) as nitrogen source or glucose (0.25%, wt/vol) as sole carbon source and choline (0.20%, wt/vol) as nitrogen source (19). Fresh medium was added at a dilution rate (D) of 0.085 per hr. Transformed strains, carrying the heterologous gene coding for glyoxysomal MDH (gMDH) from watermelon (strain GF58) or thiolase from S. cerevisiae (strain GF91) under control of the PAOX, were grown in shaker flasks in minimal medium (20) on methanol (0.5%, vol/vol) as carbon source and (NH₄)₂SO₄ (0.25%, wt/vol) or methylamine (0.20%, wt/vol) as nitrogen source.

**Plasmid Constructions.** pHIPX1 (Fig. 1A) was constructed from the following DNA fragments (numbers indicate nucleotide position on pHIPX1) (**) sticky ends filled in using Klenow enzyme; (***) sticky ends removed by mung bean nuclease**: (1-40) HindIII–Cla I(*) fragment from pTZ19R containing part of the polylinker (21). The Cla I site originates from digestion with BamHI(*) and religation. (41-340) Xho I(*)–HindIII(**) fragment containing the H. polymorpha AMO terminator region (TAMO) (22). (341-619/620-2840) contains the S. cerevisiae phosphoglycerate kinase terminator region (TPCK) and the LEU2 gene from S. cerevisiae and is identical to (442-820/821-3041) from pHIPX4 (9). (2840-4260) Bgl II–BamHI(*) H. polymorpha genomic DNA fragment present downstream of the AOX gene containing the major part of the open reading frame encoding anthranilate synthase component II (23). The BamHI site was created at the position of a Sac I site by digestion with Sac I(**) and ligation to a BamHI(*) site. (4260-6660) Cla I(*)–BamHI fragment from pH7P (24) from which the part of the multiple cloning site [HindIII(++)–Sal I(**) and religation] was deleted. (6660-8700) BamHI–HindIII H. polymorpha AOX promoter region (PAOX) (25). The HindIII site is created as described for pHIPX4 (9), pHIPX2 (Fig. 1A) was constructed analogous to pHIPX1 with some minor differences (stated below), without including the 1.4-kb Bgl II–BamHI TRP2 fragment. Coordinates: (1-45) HindIII–Sma I, polylinker pTZ19R; (45-360) Sma I–HindIII(**), T AMO; (360-640) Bgl II(*)–HindIII(**), T gK; (640-2860) HindIII(**)–BamHI(*) LEU2; (2860-5280) Cla I–BamHI, pHP7; (5280-7320) BamHI–HindIII P AOX.

A 2.2-kb HindIII–Sac I fragment encoding the AMO gene from H. polymorpha (22) was inserted in these expression vectors digested with the same restriction enzymes, resulting in plasmids pGF71 and pGF72, respectively.

A CDNA fragment encoding glyoxysomal malate dehydrogenase was cloned as a 1.3-kb Nor I(*)–Sal I (–8/1280) fragment (26) into H. polymorpha expression vector pHPX4 (9) digested with HindIII(*)–Sal I resulting in pGF92. A 1.3-kb Dde I(partial)(*)–BamHI(*) (–36/1290) DNA fragment from pRE157 (gift from W.-H. Kunau, Bochum, Germany) containing the thiolase gene from S. cerevisiae was inserted into pHIPX4 digested with HindIII(*)–Sma I, resulting in pGF224.

**Molecular Genetic Manipulation and Yeast Transformation.** Standard genetic procedures were essentially as described (27). Escherichia coli DH1 (supEs4 HsdR17 recA1 endA1 gyrA96 thi-1 relA1) was used for plasmid amplification and grown on LB medium. H. polymorpha A16 was transformed by electroporation (28). Prior to transformation the expression vectors pGF71 and pGF72 were linearized by BamHI or Sph I digestion, respectively, forcing integration of the plasmid DNA at the homologous locus on the genome (29). This results in either the addition of the plasmid-encoded expression unit in the AOX promoter locus (pGF72) or the replacement of the chromosomal AOX gene by the linearized plasmid (pGF71) (Fig. 1B). Transformants that have the plasmid integrated in the genome (integrants) were selected by growth of transformants for ~40 generations on nonselective medium. Correct integration was confirmed by Southern blot analysis (29) using the ECL direct nucleic acid labeling and detection system from Amersham. Strain GF15 contains plasmid pGF71 integrated in the genome and is therefore AOX** (methanol utilizing minus, Mut**). Strain GF16 contains plasmid pGF72 in the genome (AOX*). Expression plasmids containing pre-gMDH (pGF192) and thiolase (pGF224) were used to transform H. polymorpha A16 and were maintained as circular autonomous replicating plasmids in the host. Transformants were checked by retransformation of E. coli with total yeast DNA.

**Biochemical Methods.** Cell extracts were prepared as described (30). Protein concentrations were determined with the Bio-Rad protein assay kit using bovine serum albumin as a standard. Enzyme activities of AO (31) and AMO (32) were assayed as described and expressed as µmol of substrate consumed per min per mg of protein. The cell extracts were analyzed by SDS/PAGE (33). Gels were stained with Coomassie brilliant blue R-250 or subjected to immunoblotting (Protoblot immunoblot system; Promega) using polyclonal antibodies raised against AMO (α-AMO). Transfer of

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**Fig. 1.** (A) Physical map of the expression plasmids pHIPX1 and pHIPX2. Insertion of the H. polymorpha amine oxidase gene (AMO) yields plasmids pGF71 and pGF72. (B) Schematic representation of the chromosomal AOX locus in H. polymorpha A16 (wild type (WT)) and the transformed strains GF15 and GF16 resulting from integration of the plasmids pGF71 or pGF72 in the genome. (C) Southern analysis of H. polymorpha A16 (lane 1), GF15 (lane 2), and GF16 (lane 3). Total DNA was digested with EcoRI, and the banding pattern is a result of hybridization with a 2.3-kb BamHI–HindIII DNA fragment from pHIPX2 containing PAOX. B. BamHI; E. EcoRI; H. HindIII; Sc. Sac I; Sp. Sph I.
RESULTS

Transformation and Strain Selection. *H. polymorpha* was transformed with two different plasmids, pGF71 and pGF72. Following the isolation of integrants, two strains, GF15 and GF16, were selected by Southern blot analysis (Fig. 1C). Each of these strains contained one additional copy of the *AMO* gene under the control of the *PAOX* present in the genomic *AOX* locus. Whereas the endogenous *AMO* gene had been retained in both strains, the *AOX* gene had been deleted from the genome in strain GF15. The strains were subsequently grown under various conditions to study synthesis and peroxisomal import of *AMO*.

*PAOX*-Controlled AMO Synthesis. In glucose/(NH$_4$)$_2$SO$_4$-grown cells of strains GF15 and GF16, no AMO protein was detected. However, after shifting these cells to batch cultures containing a mixture of glycerol/methanol in the presence of (NH$_4$)$_2$SO$_4$ as sole nitrogen source, AMO activity was detected within 2 hr of incubation. As expected, WT cells contained no AMO protein under these conditions, indicating that the presence of AMO in the transformants must be due to *PAOX*-driven synthesis.

To obtain maximal expression levels of (*PAOX*-driven) heterologous AMO, the two transformants were grown in carbon-limited chemostat cultures (D = 0.085 per hr) on glucose/methanol in the presence of (NH$_4$)$_2$SO$_4$ as sole nitrogen source. In WT cells these conditions result in massive induction of *AO* constituting up to 30% of total cellular protein (38). Analysis of cell-free extracts of the chemostat-grown GF16 cells revealed the presence of high levels of AMO protein (Table 1; Fig. 2), exceeding the amount of 10% of total cellular protein. Cells of strain GF15 grown under the same conditions also synthesized AMO, although to a 4-fold lower level than observed in cells of strain GF16. The reason for this difference is unclear but may very well reflect differences in specific cultivation conditions—e.g., dissolved oxygen concentrations (39). As expected AO activity was present to WT levels in strain GF16, whereas it was invariably absent in strain GF15 (Table 1).

Cell Morphology and Subcellular Location of AMO Protein. Cells of strain GF16 contained many large peroxisomes with a largely crystalline matrix due to the crystallization of AO protein (1, 2). Peroxisomes in cells of strain GF15 were reduced in size and lacked crystalloids. Localization of AMO was examined by immunocytochemistry and immuno-EM localization (Figs. 3 and 4). The localization patterns were very similar in GF15 and GF16. However, the staining in GF16 was much stronger than in GF15.
The fact that in WT cells grown under the above conditions synthesis of AMO is fully repressed may suggest that a specific component essential for efficient import of AMO is lacking.

Efficient Import of Heterologous AMO Was Dependent on Specific Growth Conditions. WT cells of *H. polymorpha* grown in a chemostat on glucose/choline at a low D accumulate optimal levels of AO (3–4 µmol per min per mg of protein) and AMO (20–30 µmol per min per mg of protein), which are completely imported into peroxisomes (19, 40).

Under these conditions the synthesis of heterologous AMO in the transformants was also enhanced as is apparent from the AMO activity of at least 400 µmol per min per mg of protein (Table 1), which is 20-fold higher than in WT. Again, AO was normally induced to WT levels (3.5 µmol per min per mg of protein) in GF16 but was lacking in GF15. As is evident from immunocytochemical (Figs. 3 and 4), AMO was solely present in peroxisomes, irrespective of whether expressed in the AO− (GF15) or AO+ (GF16) environment.

This suggests that efficient import of AMO is dependent on a component that, together with endogenous AMO, is induced by the amine substrate.

**The Inducible Import Pathway Is Specific for PTS2 Proteins.** To determine whether the inducible import pathway is specifically involved in sorting of PTS2 proteins in general, we studied the import of two heterologous PTS2 proteins in *H. polymorpha*—namely, *S. cerevisiae* thiolase and watermelon gMDH. Previously we showed that watermelon gMDH was correctly sorted to peroxisomes in *H. polymorpha* (41). In the present study, enhanced expression levels of both heterologous genes were obtained by specific adaptations of the growth conditions of the cells. In particular, precultivation under stringent selective conditions (e.g., in the absence of yeast extract) led to enhanced protein levels, probably as a result of a higher plasmid copy number. We found that import of both heterologous proteins followed the same patterns, as described above for AMO. Thus, at high expression levels, the only part of the *S. cerevisiae* thiolase and watermelon gMDH protein was detected inside peroxisomes, whereas the remaining part accumulated in the cytosol (and nucleus) (Fig. 4 D and F). On the basis of the identical staining patterns, obtained in immunocytochemical experiments, efficient and complete import of watermelon gMDH (Fig. 4E) and bakers’ yeast thiolase (Fig. 5B), however, occurred in cells grown in the presence of methylamine as sole N source.

**DISCUSSION**

In this paper we present evidence for the existence of an inducible import pathway for peroxisomal matrix proteins with a PTS2-type targeting signal, which functions separate from the import pathway for PTS1 proteins.

The presence of different import machineries for peroxisomal matrix proteins was predicted earlier from the analysis of various peroxisome-deficient yeast strains that are impaired in the import of matrix proteins containing either a PTS1 or a PTS2 signal (12–14). The import machinery for PTS2 proteins in *H. polymorpha* appears to be inducible. This can be deduced from our data on *H. polymorpha* transformants, either expressing the homologous AMO gene or the heterologous watermelon gMDH and *S. cerevisiae* thiolase genes under the control of the *H. polymorpha* AOX promoter (*P*~*AOX*~). Efficient (and complete) import of these PTS2 proteins solely occurred when the cells were grown under conditions in which the synthesis of endogenous AMO was induced. Earlier experiments with various yeast strains have shown that the total protein storage capacity of the organelles is not fully used in normal WT cells. This is indicated by the findings that the organelles are able to import artificially overexpressed matrix giving rise to a considerable increase in organellar size (16, 42, 43). Only at very high expression levels did the import capacity become saturated, resulting in the accumulation of the additional amounts of protein in the cytosol.

Two findings argue against the possibility that such conditions are met in our present experiments. (i) All heterologous synthesized matrix proteins (AMO, watermelon gMDH, or *S. cerevisiae* thiolase) were fully imported in amine-grown
cells. (ii) In the AO- (GF16) and AO- (GF15), identical distribution patterns of heterologous AMO were observed. Furthermore, the fact that solely PTS2 proteins (but not PTS1 proteins) remain partly cytosolic in (NH4)2SO4-grown cells, even when grown in a different medium, suggests that the cytosolic import machinery is not just due to the absence of an import pathway, but also due to the presence of a specific import machinery.

The PTS1 import machinery is functional in fully repressed glucose-grown *H. polymorpha* cells as is indicated by the efficient import and assembly of AO under these conditions (42). In line with this we assume that the low-rate import of PTS2 proteins in (NH4)2SO4-grown *H. polymorpha* is due to the presence of the specific PTS2 import pathway, however at basal levels. In fact, both import machineries appear to be inducible and are in particular present at enhanced levels in cells in which the synthesis of peroxisomes is induced by specific growth substrates. Our data on *H. polymorpha* PER3, together with the findings by Subramani and coworkers (12) on *P. pastoris* PAS8, indicate that the corresponding gene products (Per3p and Pas8p, respectively) are expressed at a significantly higher level in peroxisomes compared to levels found on glucose. However, during growth of *H. polymorpha* on glucose the basal level of components involved in import of PTS1 proteins is sufficient to account for efficient import of heterologous expressed PTS1 protein. Although the (protein) nature of the components involved in the import of PTS2 proteins in *H. polymorpha* is not known at present it appears unlikely to suggest that one or a cytosolic component or a specific membranous component(s) is the limiting factor(s) in (NH4)2SO4-grown cells. Identification of the components of the PTS2 import machinery in *H. polymorpha* warrants investigation.

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