Cloning and Characterization of *PAS5*: A Gene Required for Peroxisome Biogenesis in the Methylotrophic Yeast *Pichia pastoris*

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Abstract. The biogenesis and maintenance of cellular organelles is of fundamental importance in all eukaryotic cells. One such organelle is the peroxisome. The establishment of a genetic system to study peroxisome biogenesis in the methylotrophic yeast *Pichia pastoris* has yielded many different complementation groups of peroxisomal assembly (*pas*) or peroxisome-deficient (*per*) mutants. Each appears to be deficient in functional peroxisomes. One of these mutants, *pas5*, has been characterized, complemented, and the gene sequenced. Ultrastructural studies show that normal peroxisomes are not present in *pas5*, but aberrant peroxisomal structures resembling "membranous ghosts" are frequently observed. The "peroxisome ghosts" appear to be induced and segregated to daughter cells normally. Biochemical fractionation analysis of organelles of the *pas5* mutant reveals that peroxisomal matrix enzymes are induced normally but are found mostly in the cytosol. However, purification of peroxisome ghosts from the mutant shows that small amounts (<5%) of matrix enzymes are imported. The *PAS5* gene was cloned and found to encode a 127-kD protein, which contains a 200–amino acid–long region of homology with PAS1, NEM-sensitive factor (NSF), and other related ATPases. Weak homology to a yeast myosin was also observed. The gene is not essential for growth on glucose but is essential for growth on oleic acid and methanol. The role of PAS5 in peroxisome biogenesis is discussed.

COMPARTMENTALIZATION enables the segregation of complex cellular processes in eukaryotic cells. Besides increased efficiency, compartmentalization also allows potentially harmful activities to be confined within membranous vesicles where no damage can be done to other parts of the cell. This is certainly true for the lysosome and for the peroxisome. Peroxisomes are ubiquitous, single-membrane–bound organelles that are involved in many important cellular activities. Chief among these is the oxidation of a variety of substrates by different H_2O_2-generating oxidases. Degradation of H_2O_2 is accomplished by catalase, which decomposes it to oxygen and water. Other functions in which peroxisomes are involved include bile acid synthesis (Krisans et al., 1985), plasmalogen biosynthesis (Hajra and Bishop, 1982), cholesterol metabolism (Thompson et al., 1987) and the β oxidation of long-chain fatty acids (Lazarow and De Duve, 1976). The presence of such activities is often organism, tissue, or environment specific. In addition, the peroxisome can be induced at an organellar level to proliferate in response to changing metabolic needs (Veenhuis et al., 1987).

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The importance of the peroxisome is demonstrated by the existence of various human peroxisomal disorders such as Zellweger syndrome, in which a generalized loss of peroxisomal function is found and which is believed to be due to a failure in the translocation of proteins into peroxisomes (Walton et al., 1992; Wendland and Subramani, 1993*b*). This results in the abnormal localization of peroxisomal proteins in the cytoplasm, where they cannot function, and are often rapidly turned over. The severe abnormalities that result from impairment of peroxisomal function lead to death within a few years from birth. Investigation of cell lines derived from patients with this syndrome has revealed that there are at least nine complementation groups, there being a number of genes required for function (Yajima et al., 1992; Shimozawa et al., 1992). Two of these have already been cloned and encode a 35-kD integral membrane protein called PAF1 (Shimozawa et al., 1992) and a 70-kD integral membrane protein called PMP70 (Gartner et al., 1992). In all the cell lines examined so far, there are peroxisomal "ghosts," large and apparently empty vesicles that have peroxisomal membrane proteins but are largely depleted or devoid of matrix proteins (Santos et al., 1988*a*,*b*), although there is evidence that some complementation groups may import thiolase and some other proteins (Balfe et al., 1990; Suzuki et al., 1992).

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A necessary feature of compartmentalization is the sorting of proteins to their appropriate destinations and their subsequent incorporation into organelles. All peroxisomal proteins are encoded by nuclear genes and many are known to be translocated posttranslationally into the peroxisomal membrane or matrix (For review see Subramani, 1993).

There are at least two different targeting signals involved in the transport of peroxisomal matrix proteins. Peroxisomal targeting signal 1 (PTS1)[1] consists of a carboxy-terminal tripeptide sequence that is ubiquitously used in organisms ranging from yeast to man (For review see Subramani, 1993). A second signal, PTS2, consists of an amino-terminal leader sequence that is proteolytically cleaved upon import (Swinkels et al., 1991; Osumi et al., 1991). Other signals are no doubt involved in the targeting of membrane proteins. These signals direct the proteins to the peroxisome where the translocation machinery presumably imports them. The proteins and mechanisms involved remain unknown.

To try and elucidate some of the mechanisms involved in this process, various genetic approaches have been used to isolate mutants that are deficient in peroxisomal import and/or assembly. In the yeast *Saccharomyces cerevisiae*, several mutants have been isolated and complemented (Erdmann et al., 1989, 1990; Hohfeld et al., 1991; van der Leij et al., 1992; Wiebel and Kunau, 1992). Mutants have also been isolated in the methylotrophic yeast *Hansenula polymorpha* (Cregg et al., 1990; Veenhuis, 1992). CHO cell mutants have also been generated (Zoeller and Raetz, 1986; Tsukamoto et al., 1990) and one of these was recently complemented (Tsukamoto et al., 1991) leading eventually to the isolation of a human homolog whose function was presumably deficient in a Zellweger syndrome patient (Shimozawa et al., 1992). We devised a screen that has been successful in identifying several peroxisome-defective mutants in the methylotrophic yeast *Pichia pastoris* (Gould et al., 1992). This yeast was chosen because of its ability to grow well on both oleic acid and methanol and because the metabolism of each involves two distinct and separate enzymatic pathways within the peroxisome. Growth on oleate results in the induction of several enzymes including the β oxidation enzymes (acyl-CoA oxidase, multifunctional enzyme and thiolase) and catalase. Upon growth on methanol, the peroxisomal enzymes methanol oxidase, dihydroxyacetone synthase (DHAS) and catalase are induced. By looking for cells that were unable to grow on either oleate or methanol, but that were able to use glucose, ethanol, and lactate, mutants were obtained. Confirmation of the peroxisome-defective phenotype was obtained by EM. A second feature of *P. pastoris* is its excellent morphology and amenability to EM (Hazeu et al., 1975). Wild-type cells grown on methanol or oleate have very obvious peroxisomal structures. In all of the peroxisomal mutants isolated, peroxisomes exhibiting normal morphology were not observed. Fractionation analysis was done on all of the mutants, grown under inducing conditions. They were all found to mislocalize catalase, all of the activity being found in the supernatant rather than in the pellet where the wild-type activity is found. All of the mutants obtained were recessive and were placed into eight complementation groups (Gould et al., 1992). These mutants were called peroxisomal assembly mutants (*pas*). Similar mutants (called *per* mutants) were isolated independently by Liu et al. (1992). The mutants described by the two laboratories fall into a total of 11 complementation groups (James Cregg, personal communication). One of the mutants in our collection was *pas5*. This mutant was characterized and complemented, and *PAS5* was found to be essential for peroxisomal function.

1. *Abbreviations used in this paper*: DHAS, dihydroxyacetone synthase; PAS, peroxisome assembly; PTS, peroxisomal targeting signal; SDH, succinate dehydrogenase.

Materials and Methods

Bacterial Strains

The *Escherichia coli* strain DH5α was the most frequently used strain except for plasmid rescue where we used the *E. coli* strain MC1060. Recombinant DNA techniques were performed essentially as described previously (Sambrook et al., 1989).

Yeast Strains

The wild-type strain of *Pichia pastoris* (21-1) as well as an arginine-requiring (GS190) strain (accession numbers Y-11430 and Y-18014 respectively) were obtained from the Northern Regional Research Laboratories (NRRL; Peoria, IL). For a list of strains, see Table I. The *pas5* strain was backcrossed three times. The Δ*pas5* strain was generated as described in Results. Culture, mating, sporulation, and random spore analysis were done as previously described (Gould et al., 1992).

Electron Microscopy

Electron microscopy was performed as described previously (Gould et al., 1992). However, after preculture of cells on YPD (1% wt/vol Bactopeptone, 2% wt/vol Yeast extract, 2% wt/vol Glucose), the cells were induced for 16 h in a semi-rich inducing media (1% wt/vol Bactopeptone, 2% wt/vol Yeast extract) containing either methanol (0.5% vol/vol) or oleic acid and Tween 40 (0.2% vol/vol of a 9:1 mix of oleic acid and Tween 40, respectively) before being prepared for sectioning.

Immuno-electron microscopy with rabbit antibodies against alcohol oxidase and DHAS was done as described (McCollum et al., 1993).

Fractionation of *pas5* Cells and Analysis of Proteins

Fractionation of *pas5* cells into an organellar pellet and supernatant was performed essentially as described previously (Gould et al., 1992). However, cells were grown in a semi-rich inducing media (1% Bactopeptone wt/vol, 2% wt/vol Yeast extract) containing either methanol (0.5% vol/vol–YPM) or oleic acid and Tween 40 (0.2% vol/vol of a 9:1 mix of oleic acid and Tween 40, respectively–YPOI) before being fractionated.

Fractions were assayed for catalase activity and succinate dehydrogenase (SDH) activity, as described previously (Leighton et al., 1965; Sottocasa et al., 1967). The Acyl-CoA oxidase assay was carried out as described by Hryb and Hogg (1979). Methanol oxidase activity was measured using the protocol outlined by van der Klei et al. (1990). Western blots were done using antisera to thiolase and DHAS as described in Sambrook et al. (1989). Antisera to the carboxy-terminal PTS1 peptides SKL (Gould et al., 1990) and AKI (gift of R. Rachubinski, McMaster University, Canada) were also used.

Isolation of Peroxisome Ghosts from the Δpas5 Strain

Purified peroxisomes were obtained by loading the organelle pellets of YPOI- or YPM-induced cells from the differential centrifugation assay on a sucrose gradient as previously described (Nuttley et al., 1990). Fractions were collected and assayed for catalase activity to determine the location of peroxisomes for wild-type cells. Because Δ*pas5* peroxisomes do not import catalase, gradient fractions from Δ*pas5* cells were analyzed by assaying for the presence of Acyl-CoA oxidase. Peak peroxisomal fractions collected at a density of 1.21 g/cm^3 were examined for the presence of matrix enzymes by immunoblotting also. Peroxisomal fractions from both wild-type and Δ*pas5* cells were found to be essentially free of mitochondrial contamination, determined by assaying for SDH activity (Sottocasa et al., 1967).

Table I. Strains Used

	Strain	Description
WT	PPY1	Wild-type
pas5	PPY23	*pas5* Mutant
pas5 arg	PPY200	Arginine-minus mutant
Δ*pas5*	PPY201	Disrupted strain
pas5 arg/pAPS001	PPY202	*pas5* mutant with complementing plasmid

Complementation of the pas5 Mutant and Sequencing of the Gene

Complementation of *pas5* was accomplished using reagents described previously (Gould et al., 1992). Sequencing of the complementing plasmid was performed by subcloning fragments into pBluescript/KS II (Stratagene, La Jolla, CA). Nested deletions were also generated using Exonuclease III and S1 nuclease as described previously (Sambrook et al., 1989). The Sequenase II system (United States Biochemical Corp., Cleveland, OH) which is based on the dideoxyribonucleotide chain-termination method was used for nucleotide sequencing. T3 or T7 primers were used together with denatured, double-stranded DNA templates. Both strands were entirely sequenced.

Computer Analysis of Sequences

Sequences were analyzed using the Macvector software (IBI, New Haven, CT) and also FASTA (Pearson and Lipman, 1988).

Results

The pas5 Mutant Does Not Contain Normal Peroxisomes

The *pas5* mutant was obtained by screening for the ability of mutagenized *P. pastoris* cells to grow on minimal medium containing glucose, ethanol, glycerol, or lactate as the sole carbon source but not on methanol or oleate (Gould et al., 1992).

Both wild-type and *pas5* cells were induced overnight on glucose-, oleic acid-, or methanol-containing minimal media. As previously reported (Gould et al., 1992), the wild-type cells on glucose have only a few small peroxisomal profiles (Fig. 1 *a*). On oleate, there is a dramatic increase in both number and size of the peroxisomes (Fig. 1 *c*). On methanol, the peroxisomes are also induced to proliferate but are dramatically larger than those seen in oleate-grown cells and are clustered together (Fig. 1 *e*). When *pas5* cells were examined, their appearance after growth on glucose was similar to that of wild-type cells (Fig. 1 *b*). However, after induction on oleate, there were no normal-looking peroxisomes present. Instead, some smaller, single-membrane–bound structures were present (Fig. 1 *d*), analogous to the peroxisomal ghosts seen in cell lines from patients with Zellweger syndrome (Santos et al., 1988*a*,*b*; Wiemer et al., 1989). Similar structures were observed in cells induced on methanol. Instead of peroxisomes with a large lumen characteristic of those in wild-type cells, flattened membranous structures were frequently observed, sometimes clustered in stacks (Fig. 1 *f*). These structures were often associated with the vacuolar membranes and were being engulfed by the vacuole (Fig. 2 *a*), presumably as a mechanism for degradation. The degradation of peroxisomes by lysosomes has been documented in *P. pastoris* (Tuttle et al., 1993). These structures were clearly being segregated to daughter cells and newly formed buds almost always contained these peroxisome-like structures (Fig. 2 *b*).

The pas5 Mutant Induces Peroxisomal Enzymes Normally

During growth on oleate and methanol, there is a considerable induction of the peroxisomal enzymes that are involved in the metabolism of these carbon sources. To rule out the possibility that the mutant is merely unable to appropriately induce the required enzymes for growth on oleate or methanol, we examined the induction of catalase, thiolase and DHAS.

Cells were grown on YPD to an early log stage before being transferred to minimal media containing either glucose, oleate, or methanol. After 12–18 h, these cells were spun down and cell-free extracts were made.

Catalase activity measurements revealed that in wild-type cells, there is a considerable induction of catalase when cells are grown on oleate and on methanol. In the mutant, the levels of induction on oleate and methanol were comparable, though at a slightly lower level (Fig. 3 *a*). This could be attributed to some cytoplasmic degradation or feedback in the expression levels.

Western blot analysis of wild-type and *pas5* cell-free extracts revealed that both thiolase and DHAS were induced appropriately by oleate and methanol, respectively (Fig. 3 *b*). This, together with the catalase data, demonstrates that *pas5* is not defective in the induction of peroxisomal enzymes.

The pas5 Mutant Fails to Import Most of the PTS1- and PTS2-containing Peroxisomal Proteins Synthesized

A deficiency in peroxisomal import should lead to the mislocalization of peroxisomal proteins to the cytoplasm. We tested *pas5* for its ability to target peroxisomal proteins to sedimentable structures. Cells were grown under appropriate inducing conditions and then spun down and washed. They were then spheroplasted before being homogenized by the use of a Dounce homogenizer. After removal of nuclei and cell debris, Triton X-100 (0.5% vol/vol) was added to half of the sample and then the two halves were spun at high speed to give an organellar pellet and a cytosolic supernatant. The sample with Triton X-100 added should release proteins from membranous compartments into the supernatant.

In *pas5* cells, >95% of the catalase activity was found to be in the supernatant compared with wild-type cells where the majority (~50%) of the catalase activity was in the pellet fraction (Table II). Similarly, in wild-type cells grown on methanol, 49% of the methanol oxidase activity was in the organelle pellet in a Triton-releasable form. However, none

Figure 1. pas5 cells are deficient in growth but not in proliferation of peroxisomes. (*a*) Wild-type *P. pastoris* grown in glucose medium. Mitochondria (*M*) and nucleus (*N*). (*b*) The *pas5* mutant grown in glucose medium. (*c*) Wild-type *P. pastoris* grown in oleate medium. Note numerous peroxisome-like structures (*P*), scattered throughout the cell. (*d*) *pas5* cells induced in oleate medium contain a number of small peroxisomes, as indicated by the arrow. (*e*) Wild-type *P. pastoris* grown in methanol medium. Note the presence of large clustered peroxisomes (*P*). (*f*) The *pas5* mutant induced on methanol media. Contains small peroxisome-like structures, sometimes in a stack (*arrow*). Bars, 0.5 μM.

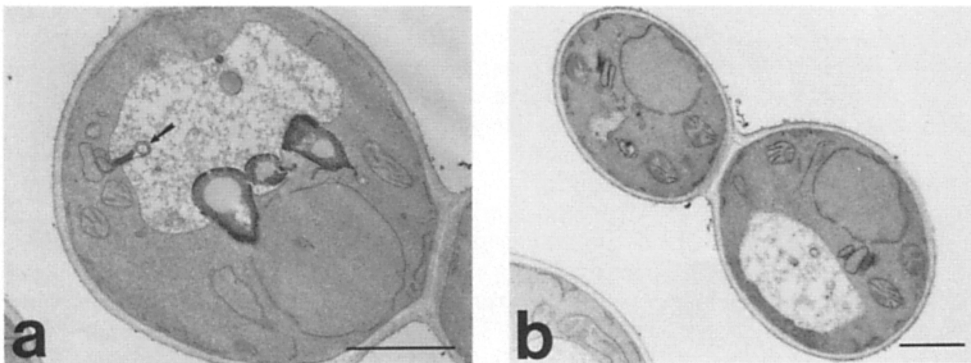
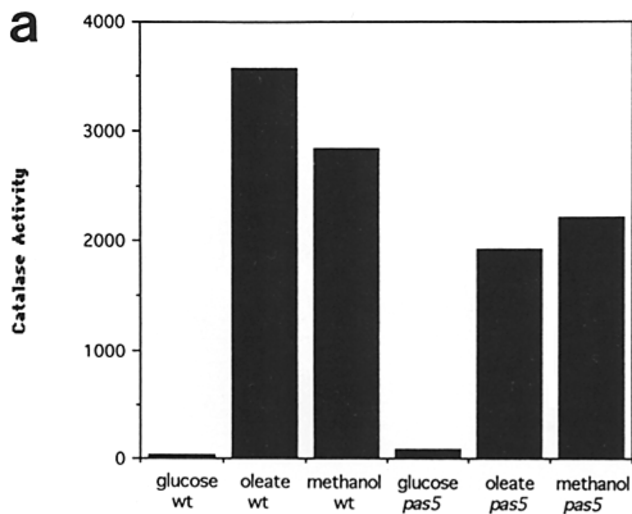


Figure 2. Peroxisome-like structures segregate and are degraded in *pas5* cells. (*a*) The peroxisome-like structures are often associated with the vacuole. In this section, there appears to be engulfment of one of these structures by the vacuolar membrane (*arrow*). (*b*) Cells were grown on methanol-containing medium. Note the stacked peroxisome-like structures in both mother and daughter cells. Bars, 0.5 μM.



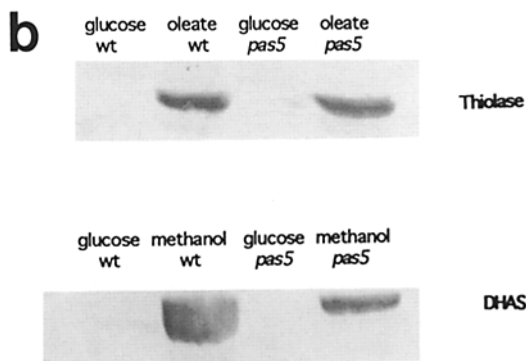


Figure 3. pas5 cells induce peroxisomal proteins. Wild-type and *pas5* cells were precultivated in rich glucose medium (YPD), and then shifted to either YPD, rich methanol medium (YPM) or rich oleate media (YPOI) for 16 h. Whole cell extracts from these cells were assayed (*a*) for catalase enzymatic activity, and by (*b*) Western blotting for the presence of DHAS protein or thiolase. Antibodies were used at dilutions of 1/200 in both cases. The amount of Catalase activity is expressed as Beaufay Units/ml.

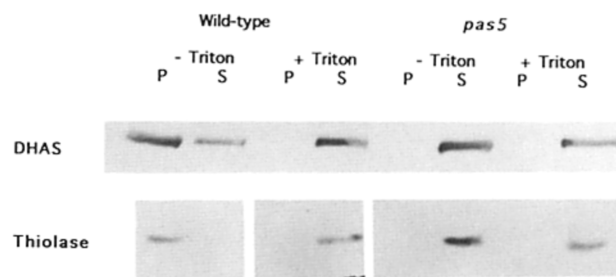


Figure 4. Thiolase and DHAS are mislocalized in *pas5* cells. Wild-type and *pas5* cells were induced for 16–18 h in either rich methanol medium (DHAS induction), or rich oleate medium (thiolase induction), and then fractionated into cytosolic supernatant (*S*), and organellar pellet (*P*) fractions after treatment with and without 0.5% Triton X-100. The pellet was resuspended in a volume equal to the supernatant and equal volumes were loaded onto the gel.

of the methanol oxidase was in the organelle pellet in the *pas5* mutant (Table II).

The localization of luciferase (which has a PTS1 sequence) was analyzed in wild-type and mutant cells. An extrachromosomal autonomously replicating plasmid (pAM2Fluc) containing the luciferase gene under the methanol oxidase promoter was introduced into the cells by electroporation. Cells were cultured in oleate and methanol-containing media. Fractionation revealed that, as expected, luciferase was found in the pellet fraction of wild-type cells and was Triton releasable. In methanol-induced wild-type cells only 15% of the luciferase was in the pellet because not all the enzyme is imported into the peroxisome when overproduced from the highly active methanol oxidase promoter (McCollum et al., 1993). In the mutant, when the *pas5* cells were grown either on oleate or methanol, the luciferase activity was almost entirely in the supernatant, which suggests that it is not imported.

When SDH activity (a mitochondrial marker enzyme) was measured, there was considerable activity in the pellet fraction of both wild-type and *pas5* cells. This indicates that the mitochondrial protein is targeted to a sedimentable structure normally in the mutant.

Western blot analysis of fractionated wild-type and *pas5* cells revealed that both thiolase (which contains PTS2) and DHAS were localized to a sedimentable organelle in wild-type cells but not in *pas5* cells (Fig. 4). In a recent study, we reported the characterization of the PAS8 protein of *P. pastoris* and showed that it was an excellent candidate for the PTS1 receptor (McCollum et al., 1993). The peroxisome-membrane-associated protein PAS8 and other peroxisomal proteins were found in the organelle pellet in *pas5* cells (see below) indicating that peroxisome-like structures might be in the pellet fractions. To further investigate the organelles in the pellet, we tried to purify peroxisome ghosts from the

Table II. Percent Total Enzyme Activity in Organelle Pellet

		Luciferase activity		Catalase activity		Methanol oxidase activity	
Strain	Carbon source	−Triton	+Triton	−Triton	+Triton	−Triton	+Triton
wt	Oleate	92	0	46	1	ND*	ND
pas5	Oleate	0	0	5	0	ND	ND
wt	Methanol	15	0	51	1	49	0
pas5	Methanol	0	0	4	0	0	0

pas5 cells not not import catalase, luciferase, and methanol oxidase. Supernatant and organellar pellet fractions were assayed for catalase activity after growth on oleate or methanol. Luciferase, containing the PTS1 sequence, was introduced to cells on a plasmid. After induction and fractionation of these cells, luciferase activity was determined in each fraction, using a luminometer. Finally, for cells grown on methanol, methanol oxidase activity was assayed (van der Klei et al., 1990). The percent of the total activities in the pellet is shown for each sample.
* Oleate grown cells do not induce methanol oxidase.

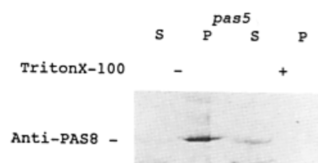


Figure 5. The PAS8 protein is associated with the pellet fraction in *pas5* cells. *pas5* cells were induced 16–18 h in rich oleate medium, and then fractionated into cytosolic supernatant (*S*), and organellar pellet (*P*) fractions after treatment with and without 0.5% Triton X-100. The pellet was resuspended in a volume equal to the supernatant and equal volumes were loaded onto the gel. Western blot analysis of these fractions using antibodies to the peroxisome membrane-associated protein PAS8 revealed that PAS8 is present in the organellar pellet of *pas5* cells. Affinity-purified PAS8 antibody used at 1/200.

pas5 null-allele strain (Δ*pas5*–for construction details, see below) to compare with wild-type peroxisomes.

Purified Peroxisomes from the Null-Allele Strain Contain a Membrane-associated Protein and Small Amounts of Peroxisomal Matrix Proteins

Since peroxisome-like structures were observed in the Δ*pas5* (see below), we decided to see if peroxisomal marker proteins were associated with these structures in the mutant. Cells were fractionated after induction on oleate-containing media and then the organelle-pellet and supernatant fractions were tested by Western blotting with affinity-purified antisera to the peroxisome-associated protein PAS8 (McCollum et al., 1993). The majority of the protein was found in the pellet fraction of *pas5* cells, as it was in wild-type cells (Fig. 5). Addition of Triton X-100 led to the loss of PAS8 signal in the pellet fraction. The presence of lower molecular weight bands is probably due to degradation of PAS8, which is particularly sensitive to proteolysis, especially in the presence of Triton X-100. This may be due to the release of a protease from a Triton X-100 sensitive compartment.

To further investigate this localization, the organellar fractions (consisting mainly of peroxisomes and mitochondria) prepared from wild-type cells and Δ*pas5* cells induced on oleate-containing medium were run on a sucrose gradient to purify peroxisomes. Catalase activity was determined and for wild-type cells, the peak activities were found in fraction 6 with a density of 1.21 g/cm^3 (Fig. 6 *a*). As expected, there was no detectable catalase activity in the Δ*pas5* fractions apart from a small amount at the top of the gradient that may be accounted for by interstitial cytosol from the pellet (Fig. 7 *a*). SDH assays were performed on the fractions of wild-type and Δ*pas5* cells and the results indicated that the peak mitochondrial activity was in fractions with a density of approximately 1.15 g/cm^3. Assays to determine Acyl-CoA oxidase activity were then performed on both sets of fractions. The activity in wild-type cells was found to colocalize with Catalase activity, peaking in fraction 6 (Fig. 6 *c*). Interestingly, this was also observed for the Δ*pas5* fractions (Fig. 7 *c*).

Equal volumes of each fraction were then taken for Western analysis and blotted with antisera to the AKI peptide which has been shown to function as a PTS in yeast (Aitchison et al., 1991). This revealed in the wild-type fractions a clear peak of protein in the region where maximal Catalase and Acyl-CoA oxidase activities had been observed. This protein was also detected in the same part of the gradient in the fractions derived from the Δ*pas5* cells.

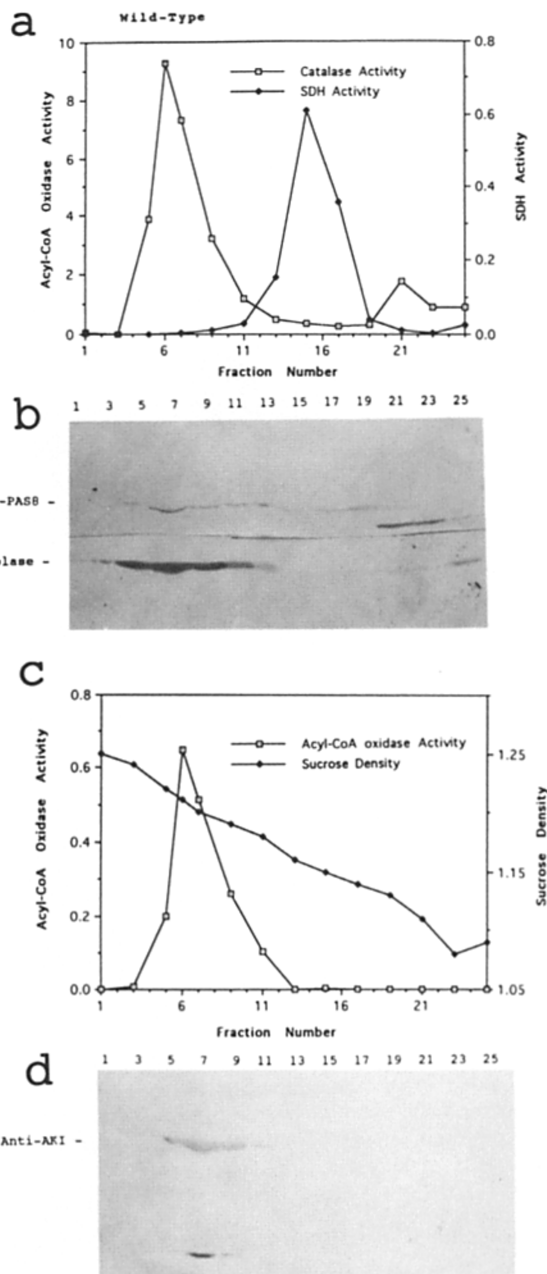


Figure 6. Purification of peroxisomes from wild-type cells on a sucrose gradient. Wild-type cells were induced on oleate for 16–18 h and the organelle pellet fraction, consisting primarily of peroxisomes and mitochondria, was fractionated on a sucrose gradient as described by Nuttley et al. (1990. (*a*) Gradient fractions assayed for catalase and SDH activity across the gradient. Catalase activity is expressed in Beaufay Units/ml. SDH activity is expressed as the change in absorbance at 410 nm/min/ml of fraction. (*b*) Equal volumes (120 μl) of each fraction analyzed by Western blotting using antisera to PAS8 and thiolase. Antibody dilution was 1/200 and 1/500, respectively. (*c*) Fractions assayed for Acyl-CoA oxidase activity. Activity measured as the increase in absorbance at 420 nm/min/ml of fraction. Density of fractions in g/cm^3. (*d*) Equal volumes (120 μl) of each fraction analyzed by Western blotting using antisera to the AKI peptide. Antibody dilution was 1/500.

Blots were then incubated with PAS8 and thiolase antisera. PAS8 was found to migrate in the gradient at densities between 1.22 g/cm^3 and 1.12 g/cm^3 in wild-type cells, peaking at ~1.21 g/cm^3 (Fig. 6 *b*). This distribution across the gra-

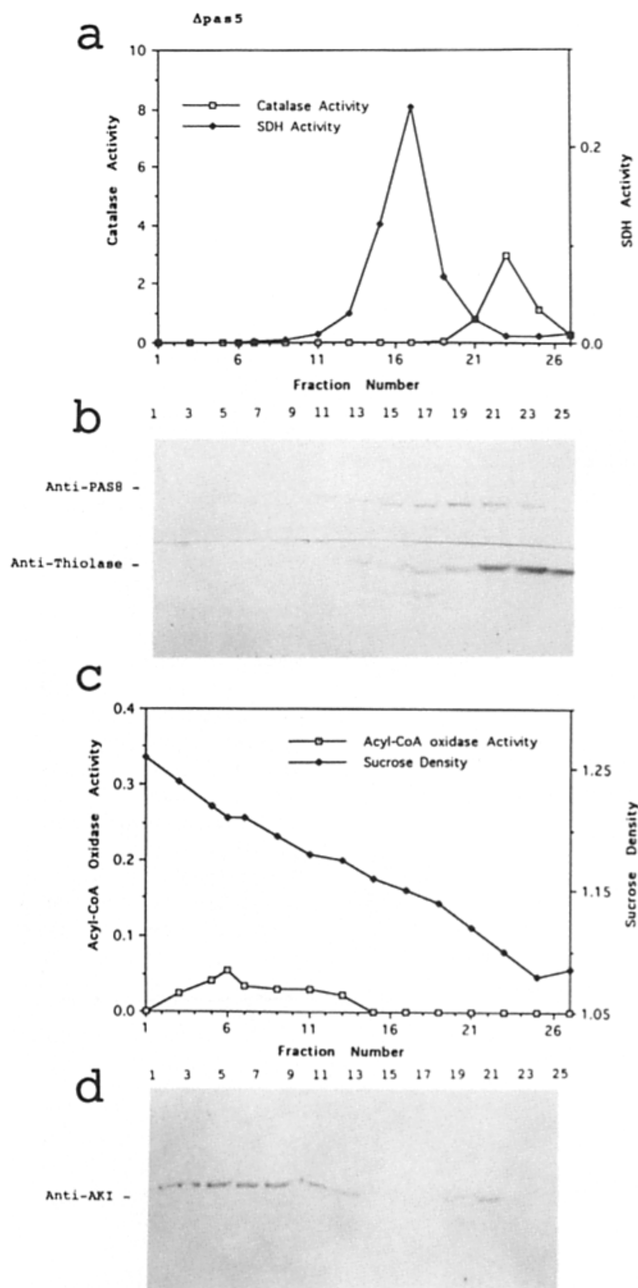


Figure 7. Purification of "peroxisome ghosts" from Δ*pas5* cells on a sucrose gradient. The organellar pellet fraction from oleate-grown Δ*pas5* cells was fractionated as described in Fig. 6. (*a*) Catalase and SDH activity across the gradient. Catalase activity is expressed in B.U./ml. SDH activity is expressed as the change in absorbance at 410 nm/min/ml of fraction. (*b*) Equal volumes (120 μl) of each fraction analyzed by Western blotting using antisera to PAS8 and thiolase. (*c*) Fractions assayed for Acyl-CoA oxidase activity. Activity measured as the increase in absorbance at 420 nm/min/ml of fraction. Density of fractions in g/cm³. (*d*) Equal volumes (120 μl) of each fraction analyzed by Western blotting using antisera to the AKI peptide.

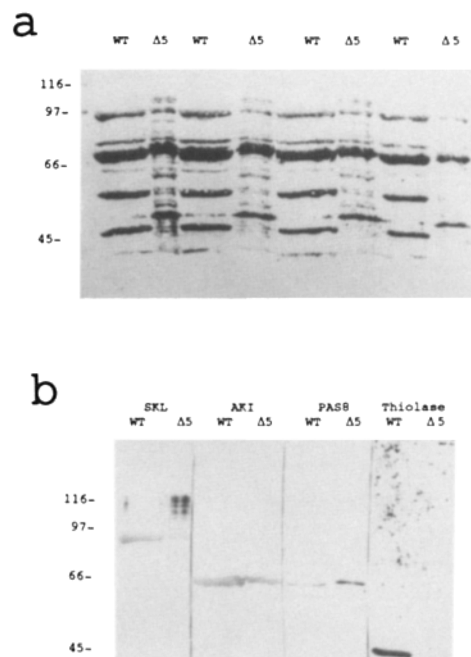


Figure 8. Comparison between peak peroxisome fractions from wild-type and Δ*pas5* gradients. (*a*) Equal amounts of protein (150 μg) of the peak fractions from wild-type (*WT*) and Δ*pas5*(*Δ5*) gradients were subjected to SDS-PAGE and then transferred to nitrocellulose. The blot was then stained by Ponceau S. The multiple of WT and Δ5 lanes, respectively, are identical. (*b*) Strips corresponding to WT and Δ5 samples in (*a*) were blotted with antisera to SKL (Antibody dilution was 1/200), PAS8 (1/500), AKI (1/200), and thiolase (1/500).

dient may be explained by the presence of peroxisomal membrane fragments produced during the homogenization procedure that contain PAS8. An alternative explanation is that there is a heterogeneous population of peroxisomes distributing at different densities. This may be similar to the case observed by Heinemann and Just (1992) who noticed that there were two distinct populations of peroxisomes that had different densities. These are thought to correspond to peroxisomes of different maturity. However, a significant proportion of the PAS8 signal colocalizes in the gradient with the matrix proteins such as catalase and Acyl-CoA oxidase, free from mitochondrial contamination, in wild-type cells. In the case of Δ*pas5* cells, PAS8 was not observed in the fractions that would normally contain it. Instead, it appears as if the majority of the PAS8 protein is present in fractions enriched for mitochondria (Fig. 7 *b*). Examination of the peak peroxisomal fraction from Δ*pas5* cells however, revealed that some PAS8 was present. Blotting across the gradients with thiolase antisera showed strong staining in the wild-type gradient at densities expected for normal peroxisomes. In the Δ*pas5* cells, staining was visible only at the top of the gradient where free protein was expected to be found (Fig. 7 *b*).

Fractions with a density of 1.21 g/cm³ where peak Acyl-CoA oxidase activities were found, were taken for both the wild-type and Δ*pas5* cells and equal amounts of protein loaded onto a protein gel. After separation by SDS-PAGE, the protein was transferred to nitrocellulose. Staining of the filter with Ponceau S revealed that a number of major proteins were present in the fractions from both wild-type and Δ*pas5* cells (Fig. 8 *a*). Some proteins were seen to be at comparable levels, others were at reduced levels or absent in the fraction from Δ*pas5* cells. The filter was then cut into four strips and blotted with antisera to SKL-peptide, PAS8, AKI-peptide, and thiolase (Fig. 8 *b*). The results show that PAS8 was present in the Δ*pas5* and wild-type fractions at comparable levels. The AKI antisera detected a major band of ~68

Figure 9. Nucleotide sequence and deduced amino acid sequence of the *P. pastoris PAS5* gene. *PAS5* nucleotide and deduced amino acid sequence. The translation of the *PAS5* sequence from the ATG, at nucleotide 1 (bold, underlined), to the termination codon is shown below the first nucleotide of each codon in the sequence. The amino acids of the protein are in the one-letter code. The ATP-binding domain of PAS5 is double underlined. Relevant restriction sites and a putative polyadenylation site are indicated. The PAS5 protein is predicted to be 1165 amino acids long with a molecular mass of 127 kD. These sequence data are available from EMBL/Genbank/DDBJ under accession number Z22556.

kD in both fractions, also at comparable levels. A smaller band of approximately 33 kD was also detected using this antisera in the wild-type peroxisomes (Fig. 6 *d*) but was not observed in the mutant (data not shown). Using the SKL antisera, a major band of ~97 kD was detected in the wild-type fraction which could be the trifunctional enzyme. This enzyme has been shown to be a major induced protein in oleate-grown cells and contains an SKL motif in *S. cerevisiae* (Hiltunen et al., 1992). This protein was also present in the Δ*pas5* fraction but at a reduced level. Finally, thiolase was present in the wild-type fraction but at barely detectable levels in the Δ*pas5* fraction. Similar studies were undertaken on methanol-induced cells and this resulted in the detection of PAS8, DHAS and methanol oxidase in fractions taken from the Δ*pas5* strain at densities of ~1.21 g/cm^3 (data not shown). Taken together, these data show that some peroxisomal proteins are present in an organellar fraction from the Δ*pas5* strain and this would further support the idea that the structures seen by electron microscopy are indeed of a peroxisomal nature. These peroxisomes can be isolated on a gradient at the appropriate density but appear to be unable to import enough matrix proteins to be functional.

The PAS5 Gene Encodes a Protein of 127 kD Related to PAS1 and Other Proteins

We cloned the *PAS5* gene by functional complementation. The mutant strain carrying the auxotrophic marker *arg4* was transformed with a genomic library and Arg$^+$ colonies were selected on minimal plates. Those colonies that contained plasmids were subsequently scored for their ability to use methanol as the sole carbon source. Those that could were checked for their ability to grow on oleate as the sole carbon source. Colonies that could use both carbon sources were grown up and DNA was isolated from them. These DNAs were then used to transform bacteria. Large-scale preparations were made and the plasmid DNA re-introduced into *pas5* cells to check that they restored the ability to use oleate and methanol. Once this was demonstrated, the DNA was sequenced and was found to contain an open reading frame of 3.5 kb that would encode a protein of 1165 aa with a predicted molecular weight of 127 kD. The sequence was examined for intron/exon splice sites but none were found. Although the Kozak sequence is weak for the first methionine in this open reading frame, we believe it to be the correct

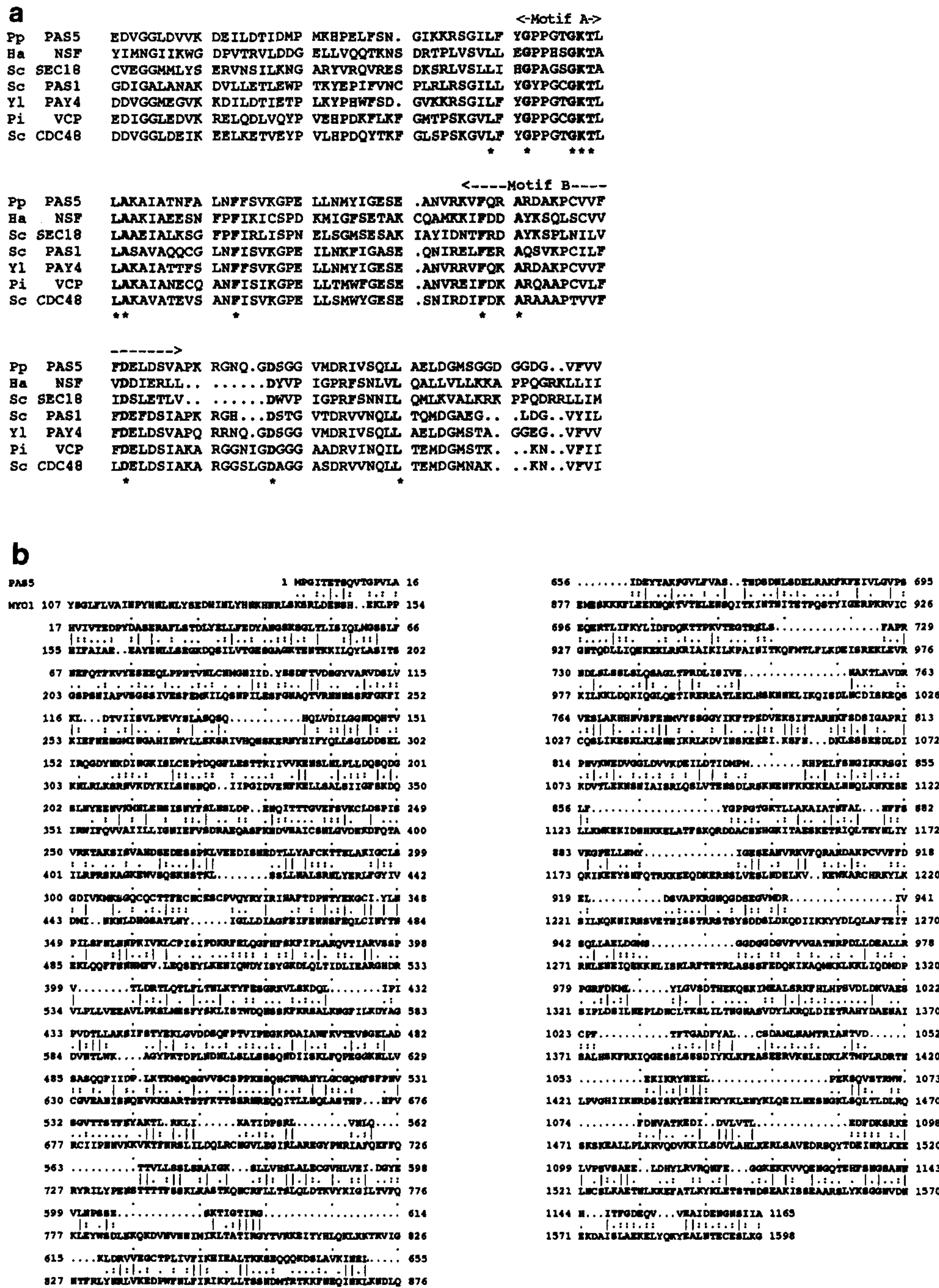


Figure 10. Comparison between PAS5 and related proteins. (*a*) Sequence alignment of protein sequences of PAS5 (*P. pastoris*), NSF (hamster), SEC18 (*S. cerevisiae*), PAS1 (*S. cerevisiae*), PAY4 (*Y. lipolytica*), CDC48 (*S. cerevisiae*), and VCP (pig). Residues conserved between all sequences are indicated by an asterisk. Motifs A and B of the consensus sequence for ATP binding are indicated. (*b*) Sequence alignment between PAS5 and Myol protein from *S. cerevisiae* using the BESTFIT program. Identities are indicated by vertical lines and similarities are indicated with dots. Two dots represents highly conserved residues and a single dot represents weakly conserved residues.

initiator for the following reasons. Firstly, a frameshift was inserted between the first and second in-frame ATGs by digestion of the DNA with BamHI, followed by fill-in of the overhanging ends with *E. coli* DNA polymerase I and ligation of the ends. This construct was then introduced into a *pas5 arg⁻* strain and found to no longer complement the inability to grow on methanol. Secondly, an antibody raised against PAS5 was used to immunoblot induced cell extracts of *P. pastoris* and it specifically recognized a protein of the expected size (data not shown).

The complete nucleotide and predicted amino-acid sequences of *PAS5* are shown in Fig. 9. The nucleic acid sequence was compared to sequences in GenBank (GenBank(R) Release 75.0, February 15, 1993) and it was found to have homologies with *PAS1* of *Saccharomyces cerevisiae* as well as other related putative ATPases including NSF (Wilson et al., 1989), SEC18 (Eakle et al., 1988), VCP (Koller and Brownstein, 1987), CDC48 (Fröhlich et al., 1991), and the human TAT-binding protein (Nelbock et al., 1990). The homology was even more pronounced at the protein level and extended over a 200–amino acid region (Fig. 10 *a*). Within this region of PAS5 was a perfect consensus ATP-binding site. The rest of the protein did not have a significant similarity to any other reported protein, except PAY4 which appears to be the PAS5 homolog in *Yarrowia lipolytica* (R. Rachubinski, personal communication). Although both PAY4 and PAS1 from *S. cerevisiae* appear to have two ATP-binding consensus sequences, the PAS5 pro-

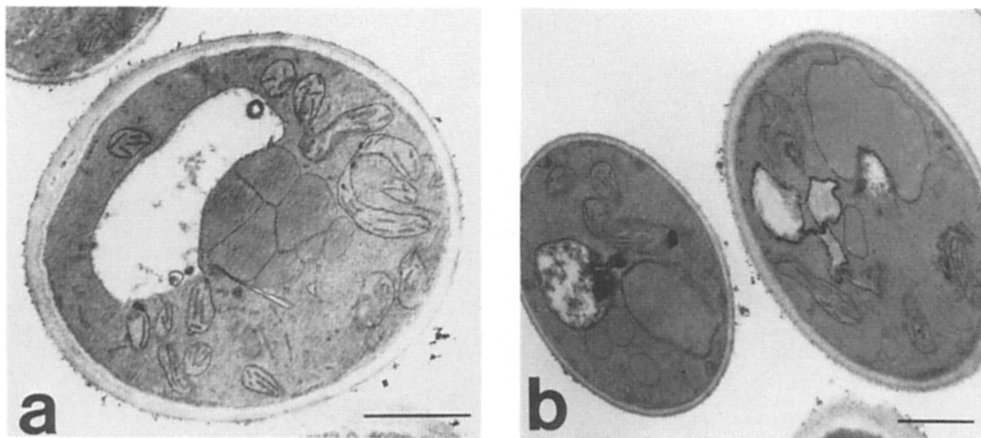


Figure 11. The *PAS5* gene restores wild-type peroxisome morphology in *pas5* cells. *pas5* cells containing the complementing plasmid pAS001 after growth in (*a*) methanol medium or (*b*) oleate medium. Bars, 0.5 μM.

tein has only a marginal second consensus sequence at amino acids 575–577. The spacing between this sequence and the full consensus sequence is the same as that found between the sequences in PAY4 and PAS1 (200–300 amino acids). It is noteworthy that in all of these proteins, the sequence similarity to other members of this ATP-binding superfamily is most pronounced at the second site.

PAS5 Shows Some Weak Homology to Myosin

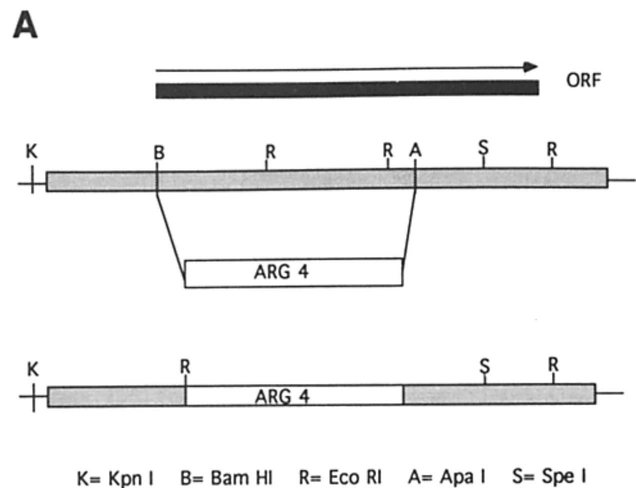
While characterizing antisera made to PAS5 protein, it was observed that there was some cross-reactivity to the 205-kD protein standard, which in this case was bovine myosin. This led us to compare the sequence of PAS5 with some myosin genes isolated from *S. cerevisiae*. The result of this comparison showed that PAS5 has ~44% similarity and 19% identity with the Myo1 protein over the entire length of the protein (Fig. 10 *b*). The Myo1 gene product is a type II heavy chain myosin believed to play a role in the budding and division of cells (Sweeney et al., 1991).

The Cloned PAS5 Gene Complements the Mutant Phenotype of pas5 Cells

The introduction of the *PAS5* gene into *pas5* cells restores their ability to grow on oleate or methanol as the sole carbon source. We then checked the morphology of *pas5* cells containing the complementing plasmid (*pas5*/pAS001) by EM. Cells were induced in minimal media containing the appropriate carbon source and then prepared in the normal way. Essentially wild-type peroxisomes were observed on both oleate and methanol as sole carbon sources (Fig. 11, *a* and *b*, respectively). A crude fractionation on these cells was also done and localization of peroxisomal matrix proteins to the pellet was observed (data not shown).

The PAS5 Gene Is Required for Normal Peroxisomal Biogenesis

A null allele of the *PAS5* gene (Δ*pas5*) was generated by integration via homologous recombination of a deletion–insertion construct. The *PAS5* gene was excised with BamHI and ApaI which eliminated 2.5 kb encoding the amino-terminal part of the gene. Then the *P. pastoris ARG4* gene was cloned into these sites (Fig. 12 *A*). The plasmid was then cut with KpnI and SpeI to yield a 3.2-kb fragment which was introduced into an *arg4* strain of *P. pastoris* by electroporation.



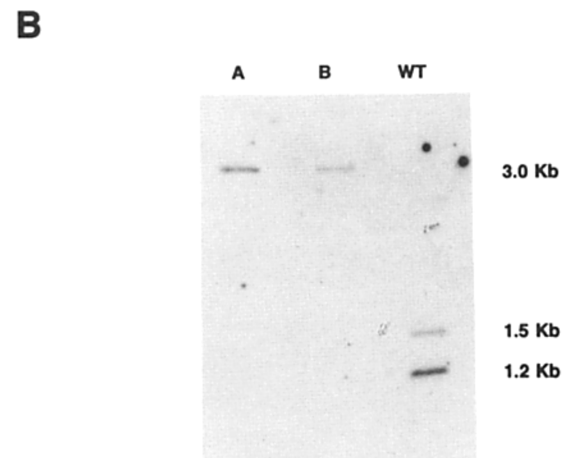


Figure 12. Disruption of the *PAS5* gene. (*A*) The *PAS5* coding region contained between the BamHI and ApaI sites was replaced with the *P. pastoris ARG4* gene and introduced into the genome by homologous recombination (see Materials and Methods for details). The sites used for generating a linear fragment and for Southern blot analysis are also shown. (*B*) Proper targeting of this construct to the genome was confirmed by Southern blotting of genomic DNA cut with EcoRI and probed with the indicated probe. Lanes *1* and *2* are two different null strains and WT is the wild-type control.

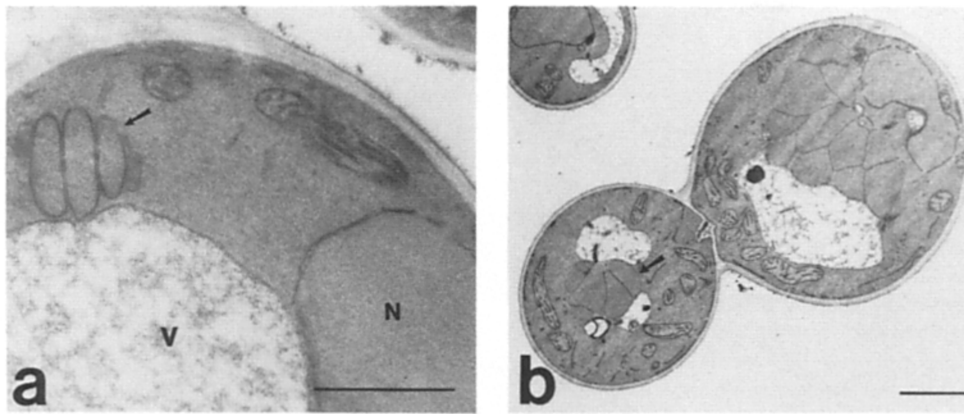


Figure 13. The null allele of *PAS5* has the same phenotype as *pas5* cells. (*a*) Δ*pas5* cells grown on methanol-containing media. In this example, the stack-like structures are shown. (*b*) Wild-type cells grown on methanol-containing medium. In the newly formed bud, the peroxisomes have not yet reached the fully mature form displayed in the mother cell. Note that the peroxisomes in this earlier stage appear to be in a similar stack-like array, before being completely filled by matrix proteins. Bars, 0.5 μM.

Cells containing the *ARG4* gene were selected and assayed for growth on methanol. Those strains that were unable to grow on methanol were checked for growth on oleate-containing media. Southern blot analysis was performed to demonstrate that the *ARG4* gene had integrated into the correct locus (Fig. 12 *B*). The stain with the deleted *PAS5* gene was mated with *pas5* cells to ensure that the right gene had in fact been cloned. The resulting diploids were still unable to use methanol or oleate as the sole carbon source, indicating that the cloned gene was indeed the *PAS5* gene.

EM was used to study the morphology of the Δ*pas5* strain. It was found to have an identical phenotype to the original *pas5* mutant. Stacked peroxisome-like structures were again found in cells induced in methanol-containing media (Fig. 13 *a*). These structures are reminiscent of the clusters of peroxisomes in newly formed buds observed in wild-type cells (Fig. 13 *b*).

Discussion

Recently, much information has emerged regarding the mechanisms of targeting and import of proteins into peroxisomes. Both biochemical and genetic studies have revealed the existence of at least two targeting signals (Gould et al., 1987, 1988, 1989; Swinkels et al., 1991) which appear to function independently to some degree (Erdmann and Kunau, 1992; McCollum et al., 1993). Less is known about the actual mechanism of import but many mutants have now been isolated in different organisms. The characterization of genes that complement these mutants and the study of the proteins they encode is likely to provide important insights regarding protein import into peroxisomes as well as the biogenesis of the organelle. The characterization of one such mutant (*pas8*) and its complementing gene from *P. pastoris* has already provided an excellent candidate for the PTS1 receptor (McCollum et al., 1993).

Peroxisome "Ghosts" in pas5 Cells Resemble Those Seen in Cells from Zellweger Syndrome Patients

We have isolated several complementation groups of mutants characterized by a general peroxisomal dysfunction. One of these is *pas5*, which does not appear to import wild-type levels of peroxisomal matrix proteins yet has some peroxisomal structures as judged by EM. These structures seem to be analogous to the "membrane ghosts" seen in patients with Zellweger syndrome (Santos et al., 1988*a*,*b*; Wiemer et al., 1989). Purification of these structures from *pas5* cells has led to the discovery that small amounts of matrix proteins are correctly localized and this is similar to the situation observed in some Zellweger cell lines. Work by the laboratories of Wanders and Hashimoto demonstrated the presence of several matrix proteins colocalizing with the peroxisomal membrane protein PMP70 (Roermund et al., 1991; Suzuki et al., 1992). Previous studies also showed that thiolase was sometimes associated with peroxisome ghost structures in Zellweger syndrome fibroblasts (Balfe et al., 1990; Gärtner et al., 1991).

As in Zellweger syndrome fibroblasts, the "peroxisome ghosts" of *pas5* cells are capable of division and segregation to daughter cells. A further similarity is that the structures in *pas5* cells are often associated with vacuolar membranes which in the yeast cell are the site of degradative and lysosomal activities. A recent report indicated that the peroxisome ghosts are themselves fused with lysosomes giving rise to autophagic vacuoles in Zellweger syndrome cells (Heikoop et al., 1992). The ghosts in Zellweger syndrome cells are larger than the wild-type peroxisomes normally seen in human fibroblasts whereas in *pas5* cells, this is not the case. These types of structures have not been described in mutants isolated from *S. cerevisiae*, where peroxisomal morphology is not as prominent as in *P. pastoris*. However, much remains to be learned about the processes that regulate peroxisome turnover and stability. For example, how does the cell discriminate between functional and nonfunctional peroxisomes with respect to turnover and degradation? In addition, the cell must preserve some progenitor peroxisome(s) that can be induced to proliferate either in response to metabolic requirements or cell cycle–dependent stimuli to ensure the segregation of organelles.

The Peroxisome Ghosts in pas5 Cells Proliferate and Segregate Normally

It is interesting to note that in *pas5* cells grown in methanol-containing media, the aberrant peroxisomes are clustered, whereas the oleate-induced structures appear to be separate, reflecting the situation observed in wild-type cells. The

clustering may be due to a specific methanol-induced membrane protein that effectively cross-links the peroxisomes, perhaps to stabilize the large peroxisomes that can result from growth on methanol. In addition, the stack-like structures suggest that the proliferating peroxisomes in methanol-grown cells may divide serially. Substantial evidence is now available to indicate that in *Candida boidinii* (Veenhuis and Goodman, 1990), and in *P. pastoris* (Heyman, J., E. Monosov, and S. Subramani, unpublished data) proliferation precedes peroxisomal protein import and organellar growth. The morphology of the peroxisomes in the *pas5* and Δ*pas5* strains is very similar to intermediate structures observed during the biogenesis of peroxisomes in a wild-type strain (Fig. 13 *b*). The biochemical data showing the normal induction of several matrix proteins in *pas5* cells and the subcellular fractionation experiments that show the most matrix proteins (catalase, luciferase, methanol oxidase, DHAS, and thiolase) are not imported into peroxisomes suggests that *pas5* cells are arrested along the normal peroxisome biogenesis pathway. The arrest appears to be between the stages of organellar proliferation and organellar growth.

The PAS5 Gene Complements the pas5 Mutant

The evidence that the cloned *PAS5* gene complements the original *pas5* mutant is as follows. The introduction of the gene into the mutant restores its ability to grow on methanol or oleic acid as the sole carbon source. It also restores the morphology to give wild-type peroxisomes on both of these carbon sources and peroxisomal protein import is normal. Construction of the null allele produces a strain indistinguishable from the original mutant in all aspects of its phenotype. Finally, matings between the Δ*pas5* and the *pas5* strains produce diploids that are still unable to metabolize methanol or oleate, genetically placing these two strains in the same complementation group. No mitotic recombinants were observed, as are frequently observed in matings of strains with different allelic mutations. This indicates that the mutation in *pas5* is most likely in the region deleted in the Δ*pas5* strain.

Data from the lab of Richard Rachubinski (personal communication) indicates that a homologue of *PAS5* has been isolated in the yeast *Yarrowia lipolytica*. In this organism, the mutant is unable to metabolize oleate as a sole carbon source. The high level of similarity between the genes, outside of the highly conserved region they share with other ATPases, leads us to believe that these are homologues and this provides evidence that the gene is conserved in other organisms.

Sequence Similarity between PAS5 and Other Proteins

Examination of the amino acid sequence of PAS5 immediately revealed a discrete region of high homology with PAS1 from *S. cerevisiae* (Erdmann et al., 1991). This is the same region that is found to be conserved amongst a family of ATPases (Fig. 10 *a*) and contains consensus sequence motifs previously described for ATP-binding proteins (Walker et al., 1982; Chin et al., 1988). This region must represent a functional domain, perhaps involved in nucleotide-regulated protein-protein interactions. NSF, which contains this domain, is known to bind to Small NSF-associated proteins (SNAPs) in an ATP-dependent fashion (Clary et al., 1990). It will be of considerable interest to determine what proteins interact with PAS5 in the cell.

Although the *PAS5* gene of *P. pastoris* exhibits some homology to the *PAS1* gene of *S. cerevisiae*, the two genes are not functional homologues of each other. The *PAS1* gene of *S. cerevisiae* is equivalent to the *PAS1* gene of *P. pastoris* (Heyman, J., and S. Subramani, unpublished data) and the *pas1* and *pas5* mutants of *P. pastoris* fall into different complementation groups.

Comparisons of PAS5 and Myosin reveals weak homology (Fig. 10 *b*) over the entire length of the protein. The significance of this is not yet clear but it is an intriguing observation.

Functional Role of the PAS5 Gene

So far, the evidence seems to indicate that there is only limited import of peroxisomal proteins into the matrix of the peroxisome-like structure of *pas5* cells. This is apparently not sufficient for growth on either oleate- or methanol-containing media. In addition, proliferation of the peroxisomal structures also appears to be normal given the reasonably large numbers of structures seen. Purification of the peroxisomes from Δ*pas5* cells has revealed that peroxisomal matrix proteins are present in the ghosts in addition to the peroxisome membrane-associated PAS8 protein. Antisera to DHAS and methanol oxidase allowed detection of these matrix proteins in the peroxisome fractions separated on sucrose gradients from methanol-induced Δ*pas5* cells. However, the amounts of these proteins present in peroxisomes of *pas5* cells is a tiny fraction of the total produced for each of these peroxisomal proteins, as judged by the fractionation results (Fig. 4 and Table II). A significant amount of AKI-containing protein and Acyl-CoA oxidase activity is present in the peroxisomes isolated from oleate-induced Δ*pas5* cells but thiolase and SKL-containing proteins are not present in significant amounts. Thus, most of the peroxisomal matrix proteins appear to be cytosolic in the mutant and Δ*pas5* cells. We could not demonstrate the presence of catalase enzyme activity in the purified Δ*pas5* peroxisomes, which could be due to the insensitivity of our assay. Alternatively, it may be present but in an inactive form, perhaps requiring other factors for its assembly into an active form.

Membrane proteins presumably play a role in proliferation and segregation of the organelle, processes that seem to be unaffected in *pas5* cells. Because *pas5* cells contain peroxisome ghosts it is possible that the localization of membrane proteins is unaffected. The presence of PAS8 protein (McCollum et al., 1993) in the purified peroxisomes of Δ*pas5* supports this idea. However, until antisera to peroxisomal integral membranes proteins are available this question cannot be resolved. This does not preclude the idea that a subset of membrane proteins is mislocalized leading to a diminution in the import of matrix proteins or a failure to enlarge the peroxisomes. We can conclude however that the *PAS5* gene product does not appear to be involved in the induction of peroxisomal proteins, organelle proliferation, or segregation.

The fact that luciferase and thiolase are not imported in the mutant indicates that both PTS1 and PTS2 pathways of peroxisomal protein import are affected in the *pas5* mutant. Luciferase has the carboxy-terminal tripeptide SKL, that was

used to define the PTS1 (Gould et al., 1987, 1988, 1989). There is mounting evidence that the other major yeast matrix proteins bear the PTS1 signal. In *S. cerevisiae* catalase ends in the tripeptide SKF and this was shown to function as the targeting signal (Kragler et al., 1993). Moreover, methanol oxidase from *P. pastoris* ends in the tripeptide ARF (Koutz et al., 1989) and DHAS from *P. pastoris* in DKL (cited in McCollum et al., 1993). Further evidence that these are all PTS1 signals comes from the characterization of the *pas8* mutant in which only the import of PTS1-containing proteins is affected, and all of the above proteins are not imported (McCollum et al., 1993). Unlike the import of thiolase into peroxisomes of *pas8* cells, this protein which contains the prototypical amino-terminal PTS2 signal is also only imported at low levels in *pas5* cells.

The apparent import of significant amounts of Acyl-CoA and a 70-kD protein recognized by antisera raised against AKI peptide may indicate that some part of the import machinery for these proteins is not so dependent on PAS5. In any case, these proteins serve as useful markers for the aberrant peroxisomal structures.

PAS5 might be involved in facilitating the import of matrix proteins containing either PTS1 or PTS2. It could serve as an accessory factor to the import machinery. The absence of PAS5 could lead to the lowered levels of proteins in the peroxisome matrix. Those proteins that are present in the matrix of Δ*pas5* cells may be imported inefficiently. Those proteins that are not detected in purified peroxisomes from Δ*pas5* cells (Fig. 8) may not be imported at all or might be degraded at a faster rate than others. Those proteins that appear unaffected for import in Δ*pas5* cells (Fig. 8) may be imported in a PAS5-independent manner.

Alternatively, the PAS5 protein may play a role in maintaining the integrity of the peroxisomal structure, allowing it to import matrix proteins, or in the growth of the organelle to accommodate new proteins. This last explanation seems unlikely since the peroxisomes clearly proliferate to some extent and would appear to be large enough to contain appreciable amounts of enzymes. Import into peroxisomes has been shown to require ATP hydrolysis (Imanaka et al., 1987; Wendland and Subramani, 1993*a*). A defect in a putative ATPase such as PAS5 might therefore be expected to affect import of matrix proteins.

Given to the weak similarity to myosin, it is tempting to speculate that PAS5 may associate with the cytoskeleton, in particular with actin. It may serve its function as an intracellular motor delivering material to developing peroxisomes, or it may play a structural role, anchoring the peroxisome to the cytoskeleton in some fashion.

We are grateful to Dr. Wolf Kunau (Ruhr University, Bochum) for his gift of anti-thiolase antibodies and to Dr. Joel Goodman (University of Texas, Southwestern Medical School, Dallas, TX) for anti-DHAS antibodies. We thank Dr. Richard Rachubinski and his colleagues (McMaster University, Ontario, Canada) for sharing information regarding the *PAY4* gene with us. We are also appreciative of the excellent technical assistance provided by Eric Wiemer and Lance Washington.

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Note Added in Proof: Dr. Henk Tabak's laboratory (University of Amsterdam, Netherlands) has recently cloned and sequenced the *S. cerevisiae PAS8* gene which appears to be a homolog of the *P. pastoris PAS5* gene (Voorn Brouwer, T., I. van der Leij, W. Hemrika, B. Distel, H. F. Tabak. 1993. *Biochem. Biophys. Acta.* In press.).

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