

# Isolation of Peroxisome Assembly Mutants from *Saccharomyces cerevisiae* with Different Morphologies Using a Novel Positive Selection Procedure

Inge Van Der Leij, Marlene Van Den Berg, Rolf Boot, Maartje Franse, Ben Distel, and Henk F. Tabak

Section for Molecular Biology, E. C. Slater Institute for Biochemical Research, University of Amsterdam, Academic Medical Centre, 1105 AZ Amsterdam, The Netherlands

**Abstract.** We have developed a positive selection system for the isolation of *Saccharomyces cerevisiae* mutants with disturbed peroxisomal functions. The selection is based on the lethality of hydrogen peroxide ( $H_2O_2$ ) that is produced in wild type cells during the peroxisomal  $\beta$ -oxidation of fatty acids. In total, 17 mutants having a general impairment of peroxisome biogenesis were isolated, as revealed by their inability to grow on oleic acid as the sole carbon source and their aberrant cell fractionation pattern of peroxisomal enzymes. The mutants were shown to have monogenetic defects and to fall into 12 complementation groups. Representative members of each complementation group were morphologically examined by immunocytochemistry using EM. In one mutant the induction

and morphology of peroxisomes is normal but import of thiolase is abrogated, while in another the morphology differs from the wild type: stacked peroxisomal membranes are present that are able to import thiolase but not catalase. These mutants suggest the existence of multiple components involved in peroxisomal protein import. Some mutants show the phenotype characteristic of glucose-repressed cells, an indication for the interruption of a signal transduction pathway resulting in organelle proliferation. In the remaining mutants morphologically detectable peroxisomes are absent: this phenotype is also known from fibroblasts of patients suffering from Zellweger syndrome, a disorder resulting from impairment of peroxisomes.

**P**EROXISOMES are cell organelles that are present in almost all eukaryotic cells. They are surrounded by a single membrane and are characterized by the presence of a fatty acid  $\beta$ -oxidation system and the enzyme catalase. Messenger RNAs encoding peroxisomal proteins are translated on free ribosomes in the cytosol and the proteins are imported posttranslationally into the organelle. Proliferation takes place by growth and division, but so far little is known about how the correct number of peroxisomes correlates with cellular demand (for reviews see Lazarow and Fujiki, 1985; Borst, 1989).

After their discovery by De Duve and Baudhuin (1966), interest in peroxisomes was primarily directed at describing their contribution to cellular metabolism, but lately there has been a growing interest in the biogenesis of the organelles. This is due to a still rudimentary knowledge of peroxisomal protein routing compared to other cellular compartments and to the existence of human genetic disorders, collectively known as the cerebro-hepato-renal or Zellweger syndrome, in which the biogenesis of peroxisomes appears to be disturbed. Patients with this syndrome have severe clinical symptoms and may die within the first year after birth (for a review see Wanders, 1988). The disease is characterized at the cellular level by a diminished number or virtual

absence of morphologically distinguishable peroxisomes. The enzymes that are normally present within the peroxisome are located in the cytosol or are missing due to premature degradation. All this suggests that protein import and/or assembly of peroxisomes are impaired in cells of patients with Zellweger syndrome and that the defects are not caused by a deficiency of a single enzyme operative in human peroxisomal metabolism. In order to increase the knowledge of peroxisome biogenesis, we have turned to the yeast *Saccharomyces cerevisiae* and followed the approach of isolating mutants with impaired peroxisomal functions. We have chosen *S. cerevisiae* since this yeast is easy to manipulate genetically. Furthermore, peroxisomes can be induced to proliferate and are essential for growth when a fatty acid, such as oleic acid, is present as the sole carbon source (Veenhuis et al., 1987).

Recently the isolation of peroxisome-deficient *S. cerevisiae* and *Hansenula polymorpha* yeast strains was reported (Erdman et al., 1989; Cregg et al., 1990). The isolation of these mutants was based on the inability of yeast cells to grow on carbon sources for which functional peroxisomes are required. As negative selection schemes are laborious, we have developed a positive selection screen for the isolation of yeast mutants with disturbed peroxisomal functions.

The system is based on the lethality of hydrogen peroxide ( $H_2O_2$ ) produced during the peroxisomal  $\beta$ -oxidation of fatty acids: the  $H_2O_2$  accumulates upon the addition of the catalase inhibitor 3-amino-1,2,4-triazol (3-AT)<sup>1</sup>. Cells that do not accumulate  $H_2O_2$  as a result of, for example, a non-functional  $\beta$ -oxidation system or ill-assembled peroxisomes are able to survive.

Using this selection scheme we have thus far isolated and characterized 11 peroxisome assembly (*pas*) mutants that fall into different complementation groups. Most mutants completely lack distinguishable peroxisomal structures, while some other mutants contain at most one or two peroxisomes per cell or a proliferated membrane structure. Furthermore, we identified a "wild-type" laboratory strain as a 12<sup>th</sup> complementation group, in which peroxisomes could not be detected. The mutant strains lacking peroxisomes resemble the Zellweger phenotype and they may prove useful as a model for the study of human peroxisomal disorders.

## Materials and Methods

### Strains, Plasmids, Media, and Culture Conditions

Strains used in this study were *S. cerevisiae* HR2 (*MAT $\alpha$* , *leu2*, *his4*, and *trp1::URA3*), BWG1-7A (*MAT $\alpha$* , *leu2*, *his3*, *ura3*, and *ade1*), and Ypl02 (*MAT $\alpha$* , *ura3-52*, *leu2- $\Delta$ 1*, *his3- $\Delta$ 200*, *ade2-101*, and *lys2-801*).

Plasmids used are Ycp50 and Ycp50-thiolase (Ycp50T), a kind gift of Dr. Kunau (University of Bochum, Germany) (Einerhand et al., 1991).

Minimal medium contained 0.67% yeast nitrogen base without amino acids (YNB) (Difco Laboratories, Detroit, MI) and amino acids (20  $\mu$ g/ml) as needed, supplemented with 2% or 0.5% (wt/vol) dextrose (SD), or 0.5% maltose (SM), or 2% (wt/vol) glycerol plus 0.05% yeast extract (Difco Laboratories) (SGY), or 0.1% (vol/vol) oleic acid, 0.5% (wt/vol) Tween-40, and 0.1% yeast extract (SOY), or 0.06% (wt/vol) lauric acid, 0.5% Tween-40, and 0.1% yeast extract (SLY), or 0.1% oleic acid, 0.06% lauric acid, 0.5% Tween-40, and 0.1% yeast extract (SOLY). Positive selection medium consisted of 0.67% YNB, 0.5% maltose, 0.06% lauric acid, 0.2% Tween-40, 0.1% yeast extract, 25 mM 3-AT, and amino acids as required (20  $\mu$ g/ml). It is important to note that the concentration of lauric acid should not exceed 0.06% since lauric acid is toxic for the cells at higher concentrations. Other media used are YPD, YPG, presporulation (GNA), and sporulation media (SPO) as described by Sherman (1991) and rich oleic acid medium (RO) containing 0.1% oleic acid, 0.2% Tween-40, 0.5% potassium phosphate buffer, pH 6, 0.3% yeast extract, and 0.5% peptone (Difco Laboratories). For plates 2% agar was added to the media described above.

Yeast cells were grown on minimal or rich media on plates or in Erlenmeyer flasks at 28°C. For the development of the positive selection screen for mutants with a disturbed  $\beta$ -oxidation system, freshly grown yeast cells were plated on different minimal plates with or without 25 mM 3-AT. After incubation for 5 d at 28°C, growth of the yeasts was determined. For cell fractionation and electron microscopic experiments the cells were extensively pregrown on liquid 0.5% SD and subsequently shifted in the exponential growth phase to RO medium. The RO culture was inoculated at an  $OD_{600}$  of 0.15 and incubated for 15 h.

### Mutagenesis and Initial Selection of Mutants

A HR2 culture grown on 2% SD was treated with 3% ethyl methyl sulfonate (EMS) as described by Fink (1970) and survival of the mutagenic treatment was determined by plating EMS-treated and control cells on YPD plates. Survival in different experiments varied between 27 and 70%. The cultures were allowed to recover from EMS treatment on 2% SD medium for about three generations and subsequently plated on positive selection plates at a concentration of  $10^7$  cells per 15-cm Petri dish. After growth for 4–5 d, colonies were picked and tested twice for inability to grow on SOY plates and ability to grow on SGY plates.

1. **Abbreviations used in this paper:** EMS, ethyl methyl sulfonate; *onu*, oleic acid non-utilizer; *pas*, peroxisome assembly; 3-AT, 3-amino-1,2,4-triazol; YNB, yeast nitrogen base.

## Cell Fractionation of Potential *Pas* Mutants

Oleic acid-induced cells were converted to protoplasts as described by Daum et al. (1982). The protoplasts were washed twice with 5 mM 2-[N-morpholino]ethane sulfonic acid (MES), pH 5.5, and 1 mM EDTA (buffer A) with 1.2 M sorbitol. The final protoplast pellet was resuspended gently in 4 ml buffer A with 0.65 M sorbitol. After lysis of protoplasts had occurred, 2.5 ml buffer A with 2.15 M sorbitol was added dropwise to the homogenate and PMSF was added to a final concentration of 1 mM. The unbroken protoplasts and nuclei were removed from the homogenate by a 10-min centrifugation step at 500 g and the resulting supernatant (S1) was centrifuged for 25 min at 20,000 g. The organellar pellet (P2) thus obtained was resuspended in buffer A with 1.2 M sorbitol and 1 mM PMSF. Marker enzymes of peroxisomes (catalase and 3-ketoacyl-CoA thiolase), mitochondria (cytochrome-*c* oxidase), and cytosol (pyruvate kinase) were measured in the S1, P2, and S2 fractions and the distribution of the enzymes over the different fractions was determined.

### Enzyme Assays

Enzyme activities were measured with a spectrophotometer (LKB Instruments Inc., Bromma, Sweden) at room temperature. Catalase activity was determined using  $H_2O_2$  as a substrate according to the method described by Lücke et al. (1963). Cytochrome-*c* oxidase activity was determined using reduced cytochrome *c* as a substrate and following the oxidation of reduced cytochrome *c* at 550 nm (Douma et al., 1985). Pyruvate kinase activity was measured in the presence of 1 mM fructose 1,6-bisphosphate as has been described by Felu et al. (1973). Multifunctional enzyme activity was assayed as described by Kionka (1985).

### SDS-PAGE/Western Blotting

SDS-PAGE was performed according to a modified version of the method of Laemmli (1970). Western blotting to nitrocellulose was performed using a semi-dry blotter from Biometra according to the instruction manual. Western blots were incubated with a polyclonal antiserum directed against denatured 3-ketoacyl-CoA thiolase (a kind gift of Dr. W. H. Kunau) and protein-antibody complexes were detected with <sup>125</sup>I-labeled protein A according to the method of Vaessen et al. (1983). Protein concentrations were measured as described by Bradford (1976) using BSA as a standard. For determination of the subcellular distribution of 3-ketoacyl-CoA thiolase, equivalent volumes of S1, P1, and S2 fractions were layered on a 12.5% SDS-PAA gel and the amounts of 3-ketoacyl-CoA thiolase in the organelle and cytosol fractions were estimated by Western blot analysis.

### EM and Immunolabeling of Cryo-thin Sections

Oleic acid-induced cells were fixed with 2% paraformaldehyde and 0.5% glutaraldehyde and ultra-thin sections were prepared as described by Gould et al. (1990). Immunolabeling of the thin sections was performed using polyclonal anti-3-ketoacyl-CoA thiolase antibodies according to the method described by Slot and Geuze (1984).

## Results

### Development of a Positive Selection Screen for Mutants with a Disturbed Peroxisomal $\beta$ -oxidation System

The positive selection system is based on the potential lethality of  $H_2O_2$  produced during the peroxisomal  $\beta$ -oxidation of fatty acids (Fig. 1). Under normal conditions  $H_2O_2$  is rapidly degraded by catalase, however addition of 3-AT inhibits catalase and the *HIS3* gene product (Kanazawa et al., 1988) in growing yeast cells. In this study we were only interested in the inhibition of catalase and therefore we used a His-wild type strain for the isolation of mutants. The use of 3-AT prevents the breakdown of  $H_2O_2$  and accumulation of this compound will lead to cell death. Cells that do not accumulate  $H_2O_2$  due, for example, to a nonfunctional  $\beta$ -oxidation system or ill-assembled peroxisomes will be able to grow.

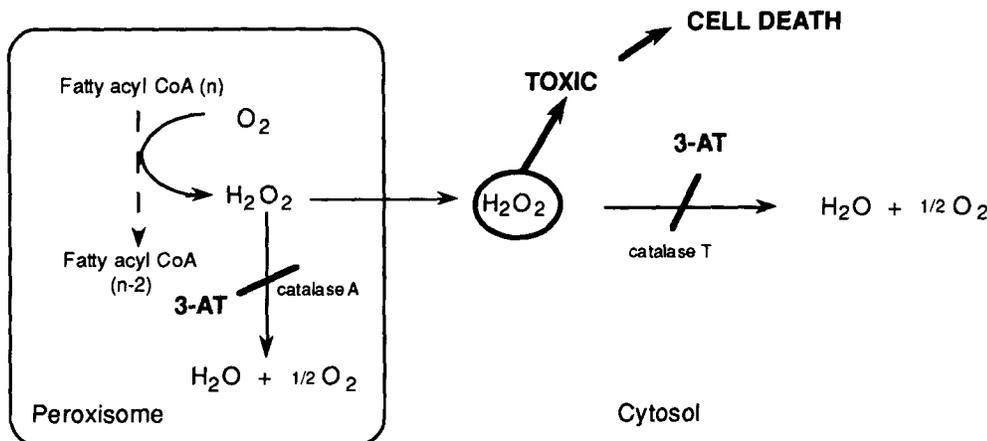


Figure 1. Peroxisomal hydrogen peroxide metabolism in yeast grown on a fatty acid.

Therefore, addition of 25 mM 3-AT, a concentration giving maximal catalase inhibition *in vivo* (results not shown), was expected to give a growth inhibition of yeast cells in the presence of a fatty acid as the sole carbon source. Growth of yeast on lauric acid, which is reported to have the greatest induction capacity of acyl-CoA oxidase and catalase (Skonecny et al., 1988), was indeed inhibited by the addition of 25 mM 3-AT (Table I). However, 3-AT was not able to suppress growth of yeast when oleic acid was present as the sole carbon source nor when both fatty acids were used in combination. This suggests that the 3-AT growth inhibition on lauric acid media is not the result of its higher induction capacity of the  $\beta$ -oxidation system leading to a higher  $H_2O_2$  production as compared to oleic acid. A more likely explanation is that the double bond present in oleic acid is capable of scavenging  $H_2O_2$ , relieving the  $H_2O_2$  stress on the cells and allowing them to grow. Yeast cells grown on oleic acid contain large cytosolic lipid droplets that might be oleic acid itself or a metabolic derivative. We presume that these lipid droplets protect the cell against peroxidative stress like plasmalogens do in higher eukaryotes (Zoeller et al., 1988). As a control we measured the effect of 3-AT on growth of yeast on maltose- or glucose-containing media. As expected, cell growth on these media was not inhibited, probably due to the absence of  $H_2O_2$  production.

The mutants with a disturbed peroxisomal  $\beta$ -oxidation system should be able to grow on a carbon source other than lauric acid in a positive selection system. Therefore the effect of addition of a second carbon source was tested on the 3-AT

growth inhibition. Various substrate and inhibitor combinations were tested. The best result was obtained using maltose as a second carbon source in the positive selection plates, despite the fact that cells grown on this medium showed a slight reduction in the amount of peroxisomal enzymes and the number of peroxisomes (see Fig. 3 b) as compared to lauric acid- or oleic acid-grown cells (see Fig. 3 a). However, normal growth is obtained when glucose (a strong repressor of peroxisome induction; Einerhand et al., 1991) is added to lauric acid and 3-AT medium. These results indicate that the selection under the conditions chosen for mutant selection is indeed based on the activity of the  $\beta$ -oxidation system.

#### Mutants with Disturbed Peroxisomal Functions Fall into 12 Different Complementation Groups

Cells recovered from treatment with EMS were plated on positive selection medium and incubated for 4–5 d at 28°C. From the colonies that appeared, 1,500 were tested for their inability to grow on plates containing minimal oleic acid medium (SOY) and their ability to grow on plates containing minimal glycerol medium (SGY) (Table II). From these, a total of 36 oleate-negative and glycerol-positive *onu* (oleic acid non-utiliser) mutants were obtained and subsequently tested for their subcellular location of the peroxisomal enzymes catalase and 3-ketoacyl-CoA thiolase in a cell fractionation experiment. The cells were harvested 15 h after a shift to oleic acid-containing medium (RO) to induce the expression of peroxisomal enzymes. In oleic acid-induced wild type cells >95% of the total catalase activity consists of the peroxisomal catalase A and only 5% is contributed by the cytosolic catalase T (Van Der Klei et al., 1990). Subcellular fractionation of wild type cells reveals that ~90% of the

Table I. Growth Behavior of Wild Type *S. cerevisiae* on Different Carbon Sources with or without 25 mM 3-AT

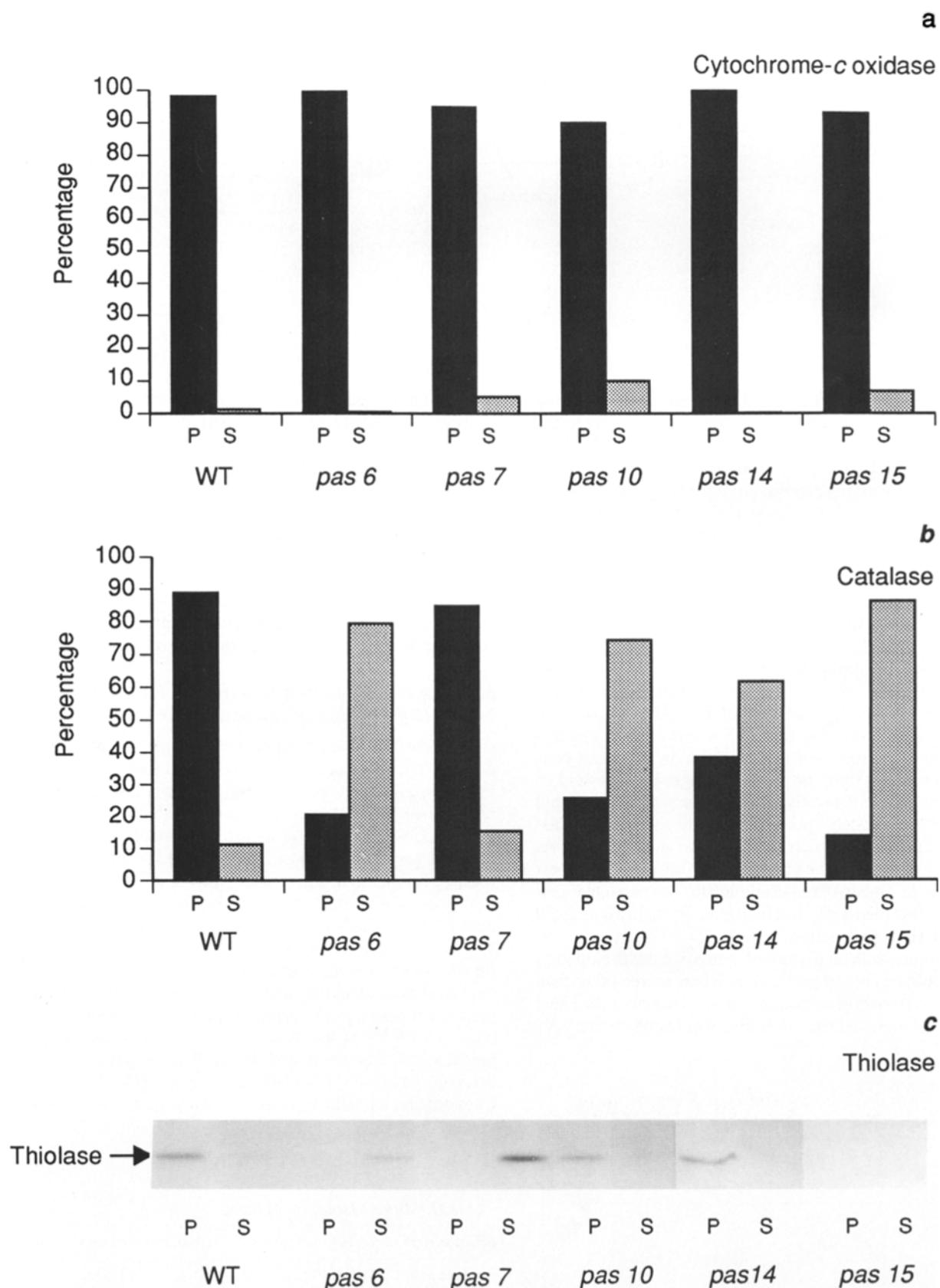
Carbon source*	Growth†	
	-3-AT	+3-AT
Glucose	+	+
Maltose	+	+
Oleic acid	+	+
Oleic acid + lauric acid	+	+
Lauric acid	+	-
Glucose + lauric acid	+	+
Maltose + lauric acid	+	-

\* Minimal medium.

† + and - indicate, respectively, normal growth and no growth.

Table II. Mutant Isolation Scheme

Selection steps after EMS mutagenesis	Number of mutants recovered
Positive selection on lauric acid, maltose, and 3-AT	1,535
Selection for oleic-acid <sup>-</sup> and glycerol <sup>+</sup> phenotype	36 (2.3%)
Aberrant cell fractionation of peroxisomal enzymes	16 (1.0%)



**Figure 2.** Distribution of marker enzymes over organellar pellet (*P*) and cytosol fraction (*S*) obtained after cell fractionation of oleic acid-induced strains. The recoveries of enzymes tested varied between 93 and 123% with a mean value of  $107\% \pm 8$ . (*a*) Distribution of cytochrome-*c* oxidase; (*b*) distribution of catalase (both were measured with spectrophotometric enzyme assays); (*c*) distribution of 3-ketoacyl-CoA thiolase (*thiolase*), measured with Western blot analysis and anti 3-ketoacyl-CoA thiolase antibodies. Equivalent amounts of cytosol and pellet fractions were layered in each lane.

**Table III. Characteristics, Deduced from Cell Fractionation Experiments and EM, of the Different *pas* Complementation Groups**

<i>pas</i> mutants*	Distribution†		
	Catalase A and T	3-ketoacyl-CoA Thiolase	Peroxisomes
<i>pas1</i> §			
<i>pas2</i> §			
<i>pas3</i> §,	C	C	Absent
<i>pas4</i> §,	C	C	Absent
<i>pas5</i> §,	ND**	ND**	Absent
<i>pas6</i> §,	C	C	Absent
<i>pas7</i> §,	P	C	Present
<i>pas8</i> §,	C	C	Absent
<i>pas9</i> §			
<i>pas10</i> §,	C	P	"reticulum"
<i>pas11</i> §			
<i>pas12</i> §			
<i>pas13</i> §			
<i>pas14</i>	C	P	1-2
<i>pas15</i>	C	P	1-2
<i>pas16</i>	C	P	1-2
<i>pas17</i>	C	P	Few, small
<i>pas18</i>	C	P	1-2

\* *pas* = peroxisome assembly deficient mutants.

† Major enzyme activity or protein found in either C (cytosol) or P (peroxisome).

§ Reviewed by Kunau and Hartig (1992).

|| Isolated by van der Leij et al. (this study).

\*\* ND = not determined.

catalase activity and >95% of the 3-ketoacyl-CoA thiolase protein is located in the organellar pellet (Fig. 2). The amount of 3-ketoacyl-CoA thiolase remaining in the supernatant presumably reflects the amount of peroxisomes broken during the fractionation experiment. As a control, the distribution of the cytochrome-*c* oxidase and pyruvate kinase over supernatant and pellet fractions was measured. More than 98% of the mitochondrial marker enzyme cytochrome-*c* oxidase sedimented in the pellet fraction, while at least 80% of the cytosolic marker remained in the supernatant (data not shown). Between different experiments the recovery of tested enzymes varied between 93 and 123% with a mean value of  $107\% \pm 8$ . 17 mutants were found to have an abnormal fractionation pattern for 3-ketoacyl-CoA thiolase and/or catalase indicating possible defects in peroxisome assembly (*pas* mutants).

These mutants were crossed with a wild type strain and the resulting diploids were able to grow on oleic acid indicating that the mutations studied are recessive. Tetrad analysis of these diploids showed a normal 2:2 segregation for the oleic acid-minus phenotype showing that these properties were caused by monogenetic defects in the nuclear genome. The mutants were further crossed with each other in all possible combinations, and also with the *pas* mutants of Erdman et al. (1989) and the *pas* mutants isolated more recently by this group. The resulting diploids were tested for growth on plates containing minimal oleic acid medium (SOY). The inability of the diploids to grow indicated that the original haploid *pas* mutants belong to the same complementation group. (Within these groups, rare cases of non-allelic non-complementation were not excluded [Stearns and Botstein, 1988]). This kind of analysis revealed that the 17 mutants with an abnormal fractionation phenotype belong to 12 different complementation groups (Table III). One of these

**Table IV. Enzyme Activities in Cell-free Extracts of Wild-Type Cells and *pas 14* Mutant Cells**

	Catalase	Multi-functional enzyme	Cytochrome- <i>c</i> oxidase
Wild type HR2	$18.3 \pm 6.3$	0.37	$0.118 \pm 0.04$
<i>pas 14</i>	$3.1 \pm 2.0$	$0.13 \pm 0.06$	$0.272 \pm 0.19$

Enzyme activities in cell-free extracts of wild type cells and of four different *pas 14* mutants that were induced on oleic acid media. Catalase, multi-functional enzyme, and cytochrome-*c* oxidase activities are expressed as, respectively,  $\Delta E_{240} \times \text{min}^{-1} \times \text{mg protein}^{-1}$ ,  $\Delta E_{307} \times \text{min}^{-1} \times \text{mg protein}^{-1}$  product formed, and  $\mu\text{mol cytochrome-}c \times \text{min}^{-1} \times \text{mg protein}^{-1}$  substrate consumed. Activities are obtained from two or four different experiments in the case of, respectively, HR2 and *pas 14*, except for the multi-functional enzyme activity of HR2 that was measured only once.

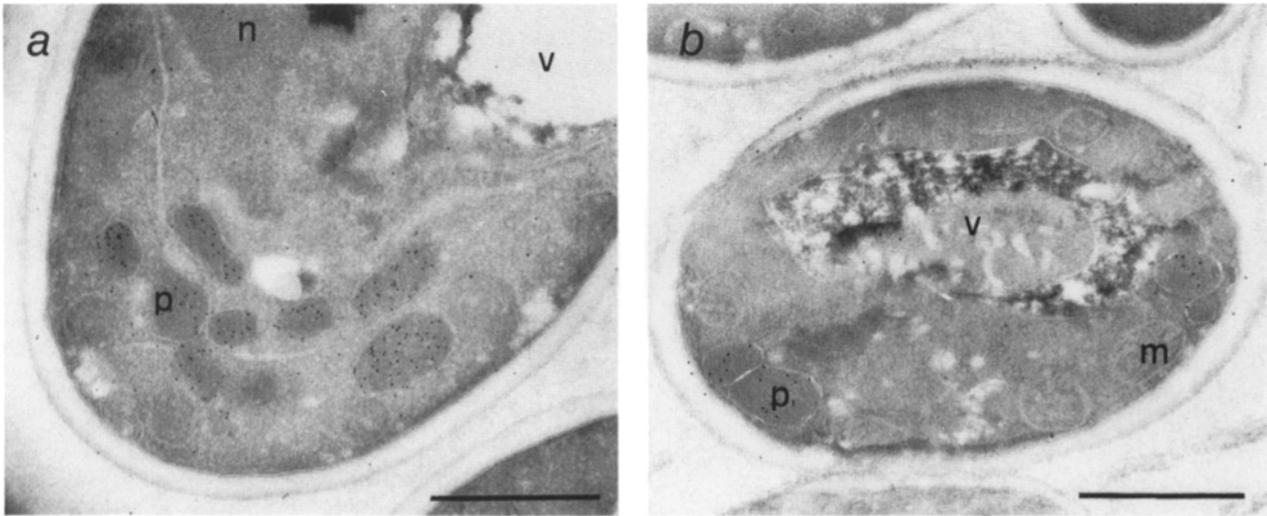
mutants was found in a different way. We noticed during cell fractionation experiments that the laboratory "wild type" strain Yp102 behaved quite differently than other wild type strains: the peroxisomal enzymes 3-ketoacyl-CoA thiolase and catalase were located predominantly in the cytosolic fraction (65–85%), while the control enzymes cytochrome-*c* oxidase and pyruvate kinase fractionated as in other wild type strains. Further analysis showed that Yp102 is a *pas* mutant representing the same complementation group as *pas 8*. Thus, the total number of complementation groups that have been found in this study is 12. One of these complementation groups has already been described (*PAS 3*; Erdman et al., 1989 and Höhfeld et al., 1991). Mutants recently found by Kunau and Hartig (1992) were made available to us for complementation analysis and are included in Table III, although the characterization of these mutants is not shown.

#### Biochemical Analysis of Mutants Based on Subfractionation of Peroxisomal Marker Enzymes

The seventeen mutants, that have an abnormal fractionation pattern for peroxisomal enzymes, display different fractionation phenotypes (Fig. 2).

First of all, the mutants *pas 3*, *4*, *6*, and *8*, among which *pas 6* is shown in Fig. 2, have both the catalase activity and the 3-ketoacyl-CoA thiolase protein predominantly located in the cytosol. The catalase and thiolase levels measured in these mutants are near wild type levels.

In contrast the levels of catalase and thiolase are decreased in the mutants *pas 14*, *15*, *16*, *17*, and *18* when compared to wild type levels. The location of the residual 3-ketoacyl-CoA thiolase protein in the *pas 14* mutant is in the peroxisomal fraction (Fig. 2). In *pas 15*, *16*, *17*, and *18* however, we have not been able to detect any thiolase protein on a Western blot. Previous studies have shown that only peroxisomal catalase A is oleic acid inducible, whereas catalase T is not (Van Der Klei et al., 1990). Thus, in mutants with low catalase activities, the cellular distribution of this enzyme probably does not reflect an import deficiency but more likely is caused by lowered amounts of peroxisomal catalase (catalase A) and unaltered amounts of cytosolic catalase (catalase T). Enzyme activity measurements in cell free extracts of four independently isolated *pas 14* mutants revealed a six- and threefold reduction in, respectively, total catalase activity and multifunctional enzyme activity whereas the mitochondrial enzyme cytochrome-*c* oxidase is not decreased (Table IV). Because catalase and thiolase are stable in the cytoplasm of other mutants (*pas 3*, *4*, *6*, and *8*) the reduced levels are probably not due to increased turnover of the proteins.



**Figure 3.** Electron microscopic pictures showing the overall cell morphology of wild type *S. cerevisiae* grown on oleic acid (a) or lauric acid and maltose (b) as carbon sources. The cryo-thin sections were immunocytochemically labeled with anti 3-ketoacyl-CoA thiolase antibodies to stain the peroxisomes. Cell organelles and cell structures are marked as follows: *p*, peroxisomes; *m*, mitochondria; *n*, nucleus; and *v*, vacuole. Bar, 1  $\mu\text{m}$ .

Therefore, these results suggest that this group of *pas* mutants is disturbed in the induction of peroxisomal enzymes, as is borne out by the *pas 14* mutant (see Discussion).

A differential alteration in peroxisomal enzyme distribution is seen in the mutants *pas 7* and *pas 10*. 3-ketoacyl-CoA thiolase is present in the organellar pellet of *pas 10*, while a normal amount of catalase activity is located in the cytosolic fraction. In *pas 7* the opposite is seen: a normal amount of catalase is located in the organellar pellet while thiolase is present in the cytosolic fraction.

#### **Electron Microscopic Analysis of the Mutants Shows Several Different, Abnormal Peroxisomal Phenotypes**

Members of each complementation group were induced on oleic acid medium and cells were analyzed under the electron microscope to determine their peroxisomal phenotype. Immunogold labeling of cryo-thin sections with anti-3-ketoacyl-CoA thiolase antibodies and protein A gold particles was used to identify peroxisomal structures. A typical example of a wild type cell is shown in Fig. 3 *a*. Gold particles stain round or oval, often clustered, vesicles with an electron-dense appearance. Representative pictures of mutant cells listed in Table III are shown in Fig. 4, *a-f*. Based on the results of the electron microscopic analysis the 12 different complementation groups can be divided into four distinct classes, ranging from morphologically normal peroxisomal profiles to total absence of detectable organelles. In the presentation of the morphological properties of the mutants the biochemical results will be taken into account.

In the mutant *pas 7*, peroxisomes are quite normal in appearance. These peroxisomes contain normal levels of catalase activity but no 3-ketoacyl-CoA thiolase protein, and therefore the peroxisomes do not label with anti-3-ketoacyl-CoA thiolase antibodies and protein A gold particles in cryo-thin sections (Fig. 4 *a*). Mutant *pas 7* cannot be complemented with the wild type 3-ketoacyl-CoA thiolase gene (Fig. 5) indicating that the mutation does not reside within the 3-ketoacyl-CoA thiolase gene itself, but affects a gene

product specifically involved in 3-ketoacyl-CoA thiolase import (a mutant that falls into this complementation group and with similar properties has been presented by Kunau at the Yeast Genetics Conference in The Hague, 1990).

Another phenotype is seen in the mutants *pas 14*, *15*, *16*, *17*, and *18*. These mutants show reduced levels of peroxisomes varying between one and a few peroxisomes per cell containing the very small amount of 3-ketoacyl-CoA thiolase that is produced (Fig. 4 *b*, *pas 14*). In addition peroxisomal enzyme levels were reduced. This phenotype resembles the glucose-repressed state in yeast with respect to peroxisome development.

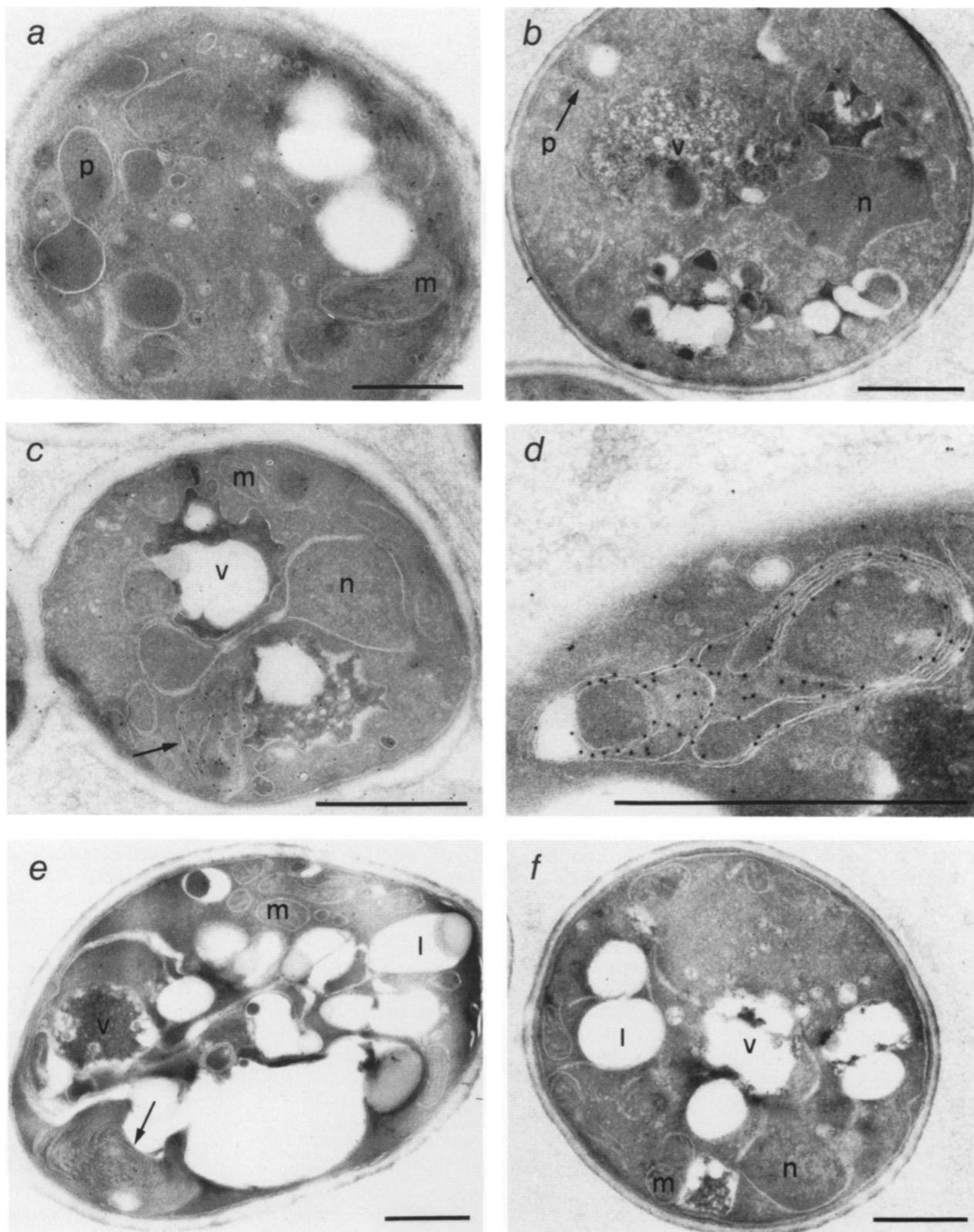
The hallmark of *pas 3* and *10* are the abnormal membrane structures, which are absent in wild type cells. The cryo-thin section of *pas 10* cells reveals a membranous reticulum with limited matrix left between the membrane layers which stains with anti-3-ketoacyl-CoA thiolase and protein A gold particles (Fig. 4, *c* and *d*). Both catalase and 3-ketoacyl-CoA thiolase are produced at wild type levels but only 3-ketoacyl-CoA thiolase is present in the membrane-bounded compartment and it is also present in a sedimentable form in a cell fractionation experiment.

The mutant *pas 3* contains a proliferated membrane structure that seems to have a close association with the lateral endoplasmic reticulum (Fig. 4 *e*, *pas 3*). Furthermore, peroxisomes are not detectable and the peroxisomal matrix enzymes are located in the cytosol.

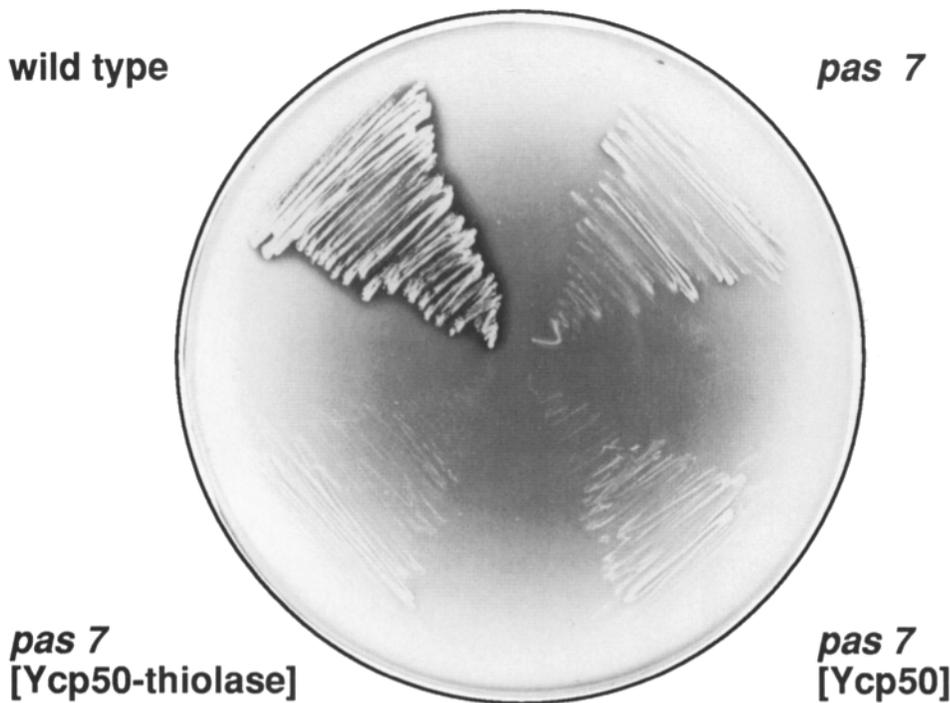
Mutants including *pas 4*, *5*, *6*, and *8*, lack morphologically distinguishable peroxisomes (Fig. 4 *f*, *pas 6*) and the peroxisomal matrix enzymes are located in the cytosol.

#### **Discussion**

We have described a novel positive selection procedure for the isolation of yeast mutants with disturbed peroxisomal functions. Using this procedure we have isolated 36 *onu* mutants disturbed in the peroxisomal  $\beta$ -oxidation, of which 16



**Figure 4.** Electron micrographs showing the overall cell morphology of representative *pas* mutants that were pregrown on glucose media and subsequently shifted to oleic acid-containing medium. All mutants were immunocytochemically labeled with anti 3-ketoacyl-CoA thiolase antibodies. (a) The peroxisomes present in *pas 7* are not labeled indicating the cytosolic location of 3-ketoacyl-CoA thiolase. (b) In *pas 14* at most one or two peroxisomes are detectable (indicated with an *arrow*) which are labeled and thus contain 3-ketoacyl-CoA thiolase. (c) *Pas 10*, labeling of a membrane structure (indicated with an *arrow*) with anti 3-ketoacyl-CoA thiolase antibodies is shown indicating its peroxisomal origin. (d) A detail is shown of the membrane structure present in *pas 10*. (e) A proliferated membrane structure is closely associated with the lateral reticulum (indicated with an *arrow*) in *pas 3*, while peroxisomes cannot be detected. (f) In *pas 6*, peroxisomes are morphologically not detectable and 3-ketoacyl-CoA thiolase is located in the cytosol. The cell organelles and cell structures are marked as follows: *p*, peroxisomes; *m*, mitochondria; *n*, nucleus; *v*, vacuole; and *l*, lipid droplet. Structures or organelles of importance are marked with arrows. Bar, 1  $\mu$ m.



**Figure 5.** Complementation analysis of *pas 7*. Transformants of the mutant *pas 7* with a plasmid containing the wild type 3-ketoacyl-CoA thiolase gene (*Ycp50-thiolase*) or a plasmid without insert (*Ycp50*) are not able to grow on a minimal oleic acid-containing plate (SOY). Untransformed *pas 7* mutant cells and wild type cells are shown as respectively a negative and a positive control on the same plate. Only wild type cells grow and show a typical "halo."

are most likely disturbed in the biogenesis of the organelle (*pas* mutants).

The frequency of *onu* mutants among the survivors of the first selection round (2:100) is 160-times higher than the frequency of *onus* found by Erdman et al. (1989) using a negative screening procedure. Notwithstanding the use of a positive selection, 95% of our surviving yeast clones were obviously not disturbed in the peroxisomal  $\beta$ -oxidation pathway. Some of these clones might have acquired resistance to 3-AT. The use of a yeast strain with a disruption of both catalase A and T as the strain for the mutagenesis in future experiments might avoid this complication.

The fact that we could impose the  $H_2O_2$  stress upon cells under the conditions used was somewhat surprising since alternative routes for removal of  $H_2O_2$  have been described. For example, cytochrome-*c* peroxidase is induced when  $H_2O_2$  is externally added to growing yeasts (Verduyn et al., 1988). We have found, however, that cytochrome-*c* peroxidase was not elevated in cells shifted to the lauric acid, maltose, and 3-AT selection condition compared to cells grown on lauric acid plus maltose or only glucose as the carbon source (data not shown). This indicates that in *S. cerevisiae* cytochrome-*c* peroxidase does not contribute significantly to the  $H_2O_2$ -degrading capacity under the selection conditions described.

Half of the originally identified 36 *onu* mutants show a normal peroxisomal phenotype, suggesting that the mutation is a single enzyme deficiency involved in either the  $\beta$ -oxidation of fatty acids itself or perhaps more indirectly in metabolite transport across the peroxisomal membrane. The 16 isolated *pas* mutants and the "wild type" strain Yp102 with a general defect in peroxisome biogenesis fall within 12 different complementation groups. The total number of genes involved in peroxisome biogenesis probably exceeds 18 (total number of *pas* complementation groups reviewed by Kunau and Hartig (1992) and found by Van Der Leij et al., Table III) since only a few alleles were found for each com-

plementation group. This indicates that it will be worthwhile screening for more mutants using this selection scheme.

In this study we have characterized mutants with an abnormal peroxisome morphology. The relative ease with which it is possible to isolate mutants with a disturbed peroxisome assembly indicates that peroxisomes are less essential for cell growth and viability than mitochondria, for instance. Peroxisomes are therefore very suitable for the study of organelle biogenesis. This is also borne out by the isolation of several different *pas* phenotypes that could result from blocks at different stages of peroxisome biogenesis.

One aspect of peroxisome formation, that takes place at an early stage, is the triggering of a regulatory cascade to induce the proliferation of peroxisomal membranes and induction of matrix enzymes in response to, for instance, the presence of oleic acid. Phenotypes that can be expected from a mutation in a regulatory cascade, such as the presence of only one or a few peroxisomes per cell, were indeed found among our mutants (*pas 14*, *15*, *16*, *17*, and *18*). Preliminary results revealed that indeed a mutation in a regulatory cascade is responsible for the phenotype of *pas 14*. The sequence of the *PAS14* gene has recently been shown to be identical to that of the *SNF1* gene (Van der Leij, I., manuscript in preparation). The *SNF1* gene encodes a protein kinase involved in the activation of glucose-repressible genes (Celenza and Carlson, 1986), e.g., genes involved in sucrose, galactose, and glycerol metabolism and also in the activation of the mitochondrial *CYCI* and *COX6* genes (Wright and Poyton, 1990). These results show that the signal transduction route regulated by the *SNF1* gene product is not only involved in the regulation of single glucose-repressible genes, but also in the regulation of the proliferation of an organelle as a whole.

In *pas 3*, an accumulated membrane structure is present that seems to have a close association with the lateral ER and peroxisomal membranes seem to be absent. Preliminary experiments revealed that these membrane structures could be

immunochemically labeled with antibodies directed against invertase, a periplasmic protein that follows the secretory pathway. These results suggest that the membrane stacks in the *pas 3* mutant originate from the ER. Taking this in conjunction with the recent observation that the *PAS 3* gene encodes a peroxisomal membrane protein (Höhfeld et al., 1991), it is tempting to speculate that the *PAS 3* protein functions as a receptor directing phospholipid transport from the ER to the peroxisomes.

Another basic process in peroxisome formation is the import of newly synthesized proteins into peroxisomes. Two of our *pas* mutants are possibly disturbed at this stage of peroxisome assembly: in the first of these, *pas 7*, peroxisomes are formed normally but the matrix enzyme 3-ketoacyl-CoA thiolase is not imported into the organelles while catalase is. The wild type 3-ketoacyl-CoA thiolase gene is not able to complement *pas 7*, suggesting that the mutation does not reside within the 3-ketoacyl-CoA thiolase gene itself but affects a gene product specifically involved in 3-ketoacyl-CoA thiolase import. The recent description of an NH<sub>2</sub>-terminal import signal in peroxisomal rat 3-ketoacyl-CoA thiolase distinct from the SKL motif (Gould et al., 1990; Swinkels et al., 1991; Osumi et al., 1992) supports the notion that yeast 3-ketoacyl-CoA thiolase may enter the peroxisomes via a different route.

The other mutant with a possible import defect, *pas 10*, is characterized by the presence of a reticulum-like membrane structure that contains 3-ketoacyl-CoA thiolase whereas the other tested matrix enzyme, catalase, is located in the cytosol. Preliminary experiments show that also luciferase, a marker protein for the SKL import pathway, expressed from a plasmid, is not imported into these membranes. This could indicate a disturbance in a component of the import machinery, which is normally involved in the import of both catalase and luciferase but not 3-ketoacyl-CoA thiolase.

Finally, mutants were found in which peroxisomal structures were morphologically and biochemically absent (*pas 4, 5, 6, and 8*). Genes with mutations giving rise to such phenotypes could be involved in peroxisome formation, division, segregation to daughter cells or even peroxisome turnover. The laboratory strain without any morphologically detectable peroxisomes also belongs to this group of mutants. Apparently standard laboratory conditions do not always require functional peroxisomes.

The *pas* mutants described in this paper reveal different stages of peroxisome assembly. The phenotypes of yeast cells showing a total absence of peroxisomes or the presence of peroxisomes at quite reduced levels resemble the phenotypes of cells isolated from patients suffering from the Zellweger syndrome. The phenotype of *pas 7*, which contains peroxisomes that contain at least catalase but not 3-ketoacyl-CoA thiolase, resembles another human peroxisome disorder, the Rhizomelic form of *Chondrodysplasia punctata* (Wanders et al., 1988). These yeast mutants may therefore prove useful as model systems for the study of human peroxisome disorders and will undoubtedly add to our present knowledge of the different aspects of peroxisome biogenesis.

We wish to thank Professor J. M. Tager and Dr. S. Brul for suggesting to us the application of the H<sub>2</sub>O<sub>2</sub> suicide technique for isolation of mutants. We are very grateful to Professor W. H. Kunau and collaborators for the possibility to use their *pas* mutants in our complementation analysis, the gift of the 3-ketoacyl-CoA thiolase antibody, and the plasmid containing the wild type

thiolase gene, and for the stimulating discussions on the results prior to publication. Thanks are also due to W. Kos for performing the initial experiments on the development of the mutant isolation screen, to T. Voorn-Brouwer for the isolation of some of the mutants, and to A. Kal for experimental support. We would like to thank Professor P. Borst, Dr. R. Benne, Dr. P. Sloof, and Dr. P. Weijers for their interest in our work.

This work was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Foundation for Scientific Research (NWO) and the Biotechnology Centre Amsterdam.

Received for publication 2 December 1991 and in revised form 20 May 1992.

*Note Added in Proof.* Recently we have obtained evidence that *pas 14, pas 15, and pas 17* belong to the same complementation group. This means that we have isolated only 10 different complementation groups instead of 12 as reported above. The separate identity of the remaining complementation groups has been confirmed by various independent criteria at our lab and that of Kunau's.

## References

- Borst, P. 1989. Peroxisome biogenesis revisited. *Biochim. Biophys. Acta.* 1008:1-13.
- Bradford, M. M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Celenza, J. M., and M. Carlson. 1986. A yeast gene that is essential for release from glucose repression encodes a protein kinase. *Science (Wash. DC).* 233:1175-1180.
- Cregg, J. M., I. J. Van Der Klei, G. J. Sulter, M. Veenhuis, and W. Harder. 1990. Peroxisome-deficient mutants of *Hansenula polymorpha*. *Yeast.* 6:87-97.
- Daum, G., P. C. Böhni, and G. Schatz. 1982. Import of proteins into mitochondria. Cytochrome *b2* and cytochrome-*c* peroxidase are located in the intermembrane space of yeast mitochondria. *J. Biol. Chem.* 257:13028-13033.
- De Duve, C., and P. Bauduin. 1966. Peroxisomes (Microbodies and related particles). *Physiol. Rev.* 46:323-357.
- Douma, A. C., M. Veenhuis, W. De Koning, M. Evers, and W. Harder. 1985. Dihydroxyacetone synthase is localized in the peroxisomal matrix of methanol grown *Hansenula polymorpha*. *Arch. Microbiol.* 143:237-243.
- Einerhand, A. W. C., T. M. Voorn-Brouwer, R. Erdman, W. H. Kunau, and H. F. Tabak. 1991. Regulation of transcription of the gene coding for peroxisomal 3-oxoacyl-CoA thiolase of *Saccharomyces cerevisiae*. *Eur. J. Biochem.* 200:113-122.
- Erdman, R., M. Veenhuis, D. Mertens, and W. H. Kunau. 1989. Isolation of peroxisome-deficient mutants of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA.* 81:5419-5423.
- Felfu, J. E., L. Hue, and H. G. Hers. 1976. Hormonal control of pyruvate kinase activity and of gluconeogenesis in isolated hepatocytes. *Proc. Natl. Acad. Sci. USA.* 73:2762-2766.
- Fink, G. R. 1970. The biochemical genetics of yeast. In *Methods of enzymatic analysis*. Vol. 17. Tabor, H. and C. W. Tabor, editors. Academic Press, Inc., New York and London. 59-78.
- Gould, S. J., G. Keller, M. Schneider, S. Howell, L. Garrard, J. M. Goodman, B. Distel, H. F. Tabak, and S. Subramani. 1990. Peroxisomal protein import is conserved between yeast, plants, insects and mammals. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:85-90.
- Höhfeld, J., M. Veenhuis, and W. H. Kunau. 1991. *PAS3*, a *Saccharomyces cerevisiae* gene encoding a peroxisomal integral membrane protein essential for peroxisome biogenesis. *J. Cell Biol.* 114:1167-1178.
- Kanazawa, S., M. Driscoll, and K. Struhl. 1988. *ATR1*, a *Saccharomyces cerevisiae* gene encoding a transmembrane protein required for aminotriazol resistance. *Mol. Cell Biol.* 8:664-673.
- Kionka, C., and W. H. Kunau. 1985. Inducible  $\beta$ -oxidation pathway in *Neurospora crassa*. *J. Bact.* 161:153-157.
- Kunau, W. H., and A. Hartig. 1992. Peroxisome biogenesis in *Saccharomyces cerevisiae*. In *Antonie Van Leeuwenhoek*. 62:63-78.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Lazarow, P. B., and Y. Fujiki. 1985. Biogenesis of peroxisomes. *Annu. Rev. Cell Biol.* 1:489-530.
- Lücke, H. 1963. Catalase. In *Methods of enzymatic analysis*. H. U. Bergmeier, editor. Academic Press, Inc., New York. 885-894.
- Osumi, T., T. Tsukamoto, S. Hata, S. Yokota, S. Miura, Y. Fujiki, M. Hijikata, S. Miyazawa, and T. Hashimoto. 1992. Amino-terminal presequence of the precursor of peroxisomal 3-ketoacyl-CoA thiolase is a cleavable signal peptide for peroxisomal targeting. *Biochem. Biophys. Res. Commun.* 181:947-954.
- Sherman, F. 1991. Getting started with yeast. In *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*. Guthrie, C., and G. R. Fink, editors. Academic Press, Inc., San Diego, California. 194:3-21.
- Skoneczny, M., A. Chelstowska, and J. Rytko. 1988. Study of the coinduction by fatty acids of catalase A and acyl-CoA-oxidase in standard and mutant

- Saccharomyces cerevisiae* strains. *Eur. J. Biochem.* 174:297-302.
- Slot, J. W., and H. J. Geuze. 1981. In Immunolabeling for Electron Microscopy. Polak, J. M., and I. M. Varndell, editors. Elsevier, Amsterdam. 129-142.
- Stearns, T., and D. Botstein. 1988. Unlinked noncomplementation: isolation of new conditional-lethal mutations in each of the tubulin genes of *Saccharomyces cerevisiae*. *Genetics.* 119:249-260.
- Swinkels, B. W., S. J. Gould, A. G. Bodnar, R. A. Rachubinski, and S. Subramani. 1991. A novel, cleavable peroxisomal targeting signal at the amino-terminus of the rat 3-ketoacyl-CoA thiolase. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3255-3262.
- Vaessen, R. T. M. J., E. Van Eijk, and L. A. Grivell. 1983. Protein transfer to nitrocellulose filters. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 124:193-196.
- Van Der Klei, I. J., J. Rytka, W. H. Kunau, and M. Veenhuis. 1990. Growth profiles and catalase activities in relation to microbody proliferation. *Arch. Microbiol.* 153:513-517.
- Veenhuis, M., M. Mateblowsky, W. H. Kunau, and W. Harder. 1987. Proliferation of microbodies in *Saccharomyces cerevisiae*. *Yeast.* 3:77-84.
- Verduijn, C., M. L. F. Giuseppin, W. A. Scheffers, and J. P. Van Dijken. 1988. Hydrogen peroxide metabolism in yeasts. *Appl. Environ. Microbiol.* 54:2086-2090.
- Wanders, R. J. A., H. S. A. Heymans, R. B. H. Schutgens, P. H. Barth, H. Van Den Bosch, and J. M. Tager. 1988. Peroxisomal disorders in neurology. *J. Neurol. Sci.* 88:1-39.
- Wright, R. M., and R. O. Poyton. 1990. Release of two *Saccharomyces cerevisiae* cytochrome genes, *COX6* and *CY1*, from glucose repression requires the *SNF1* and *SSN6* gene products. *Mol. Cell. Biol.* 10:1297-1300.
- Zoeller, R. A., O. H. Morand, and C. R. H. Raetz. 1988. A possible role for plasmalogens in protecting animal cells against photosensitized killing. *J. Biol. Chem.* 263:11590-11596.