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Peroxisome Biogenesis and Degradation in Yeast: A Structure/Function Analysis

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ABSTRACT In yeast, peroxisomes are the site of specific catabolic pathways that characteristically include hydrogen peroxide producing oxidases and catalase. During the last 10 years, much progress has been made in unravelling the molecular mechanisms involved in the biogenesis of this organelle. At present, 23 different genes (*PEX* genes) have been identified that are involved in different aspects of peroxisome biogenesis (e.g., proliferation, formation of the peroxisomal membrane, import of matrix proteins). The principles of peroxisome degradation are still much less understood. Recently, the first yeast mutants affected in this process have become available and used to clone corresponding genes by functional complementation. In this paper, an overview is presented of the research on yeast peroxisomes, focusing on recent achievements in the molecular aspects of peroxisome development, function, and turnover. *Microsc. Res. Tech. 51:584–600, 2000.*

INTRODUCTION

Microbodies (peroxisomes, glyoxysomes, and glycosomes) are essential organelles that constitute the last class of cell compartments to be discovered (in 1954). The organelles consist of a single membrane that encloses a proteinaceous matrix and measure up to 1 μm in diameter. Despite their simple morphology, the functional diversity of these organelles is unprecedented. These functions range from crucial roles in the β-oxidation of very long chain fatty acids and biosynthesis of cholesterol in man, mobilisation of storage oil and photorespiration in plant, glycolysis in trypanosomes (the organisms that cause sleeping sickness), penicillin production in filamentous fungi to functioning in the primary metabolism of various carbon and/or nitrogen sources in yeast (reviewed by Van den Bosch et al., 1992; Veenhuis and Harder, 1988). Their importance in cellular metabolism was emphasized by the discovery of a series of inherited peroxisomal disorders in man, which generally cause an early death. This finding, that peroxisomal function is essential for human life, has strongly stimulated research into microbodies.

During the last 10 years, much progress has been made in research on microbody function, biogenesis, and degradation. Major breakthroughs have resulted from detailed analyses of yeast mutants that are defective in microbody biogenesis or degradation and the use of these mutants to clone corresponding genes by functional complementation. To date, 23 genes involved in peroxisome biogenesis (*PEX* genes) have been cloned and analysed. Moreover, the first reports on genes involved in peroxisome degradation in yeasts (*GSA1, GSA7, PDD1* and *VPS15*) were recently published. In this contribution, relevant new achievements in peroxisome biogenesis and selective turnover are summarised.

PEROXISOME PROLIFERATION AND TURNOVER IN YEAST

A characteristic feature of yeast peroxisomes is that they are inducible. The organelles rapidly develop during growth of cells in media containing specific carbon and/or nitrogen sources (e.g., alkanes, fatty acids, methanol, uric acid, or primary amines; for review see Veenhuis and Harder, 1988). Under these conditions, peroxisomes are essential for growth. Their shape, ultimate number, and volume fraction is largely determined by the growth conditions, i.e., the choice of the substrate and the growth rate (Fig. 1). So far, highest peroxisomal abundances have been encountered in methylotrophic yeast (e.g., *Candida boidinii, Hansenula polymorpha, Pichia pastoris*; see Fig. 5A). When grown in a methanol-limited chemostat at low dilution rates, up to 80% of the total cytoplasmic volume may be occupied by peroxisomes (Veenhuis et al., 1978). These organelles contain the key enzymes involved in methanol-metabolism (alcohol oxidase [AO], dihydroxyacetone synthase [DHAS], and catalase) and show a completely crystalline substructure due to the crystallisation of AO protein (Figs. 1, 2).

The kinetics of peroxisome development have been studied in detail in *H. polymorpha*. Glucose-grown cells of this organism characteristically contain one or very few small peroxisomes. After a shift of such cells into fresh methanol-containing media, these organelles serve as the target for the newly synthesised peroxisomal enzymes involved in methanol metabolism (AO, DHAS, and catalase). As a result of the uptake of these

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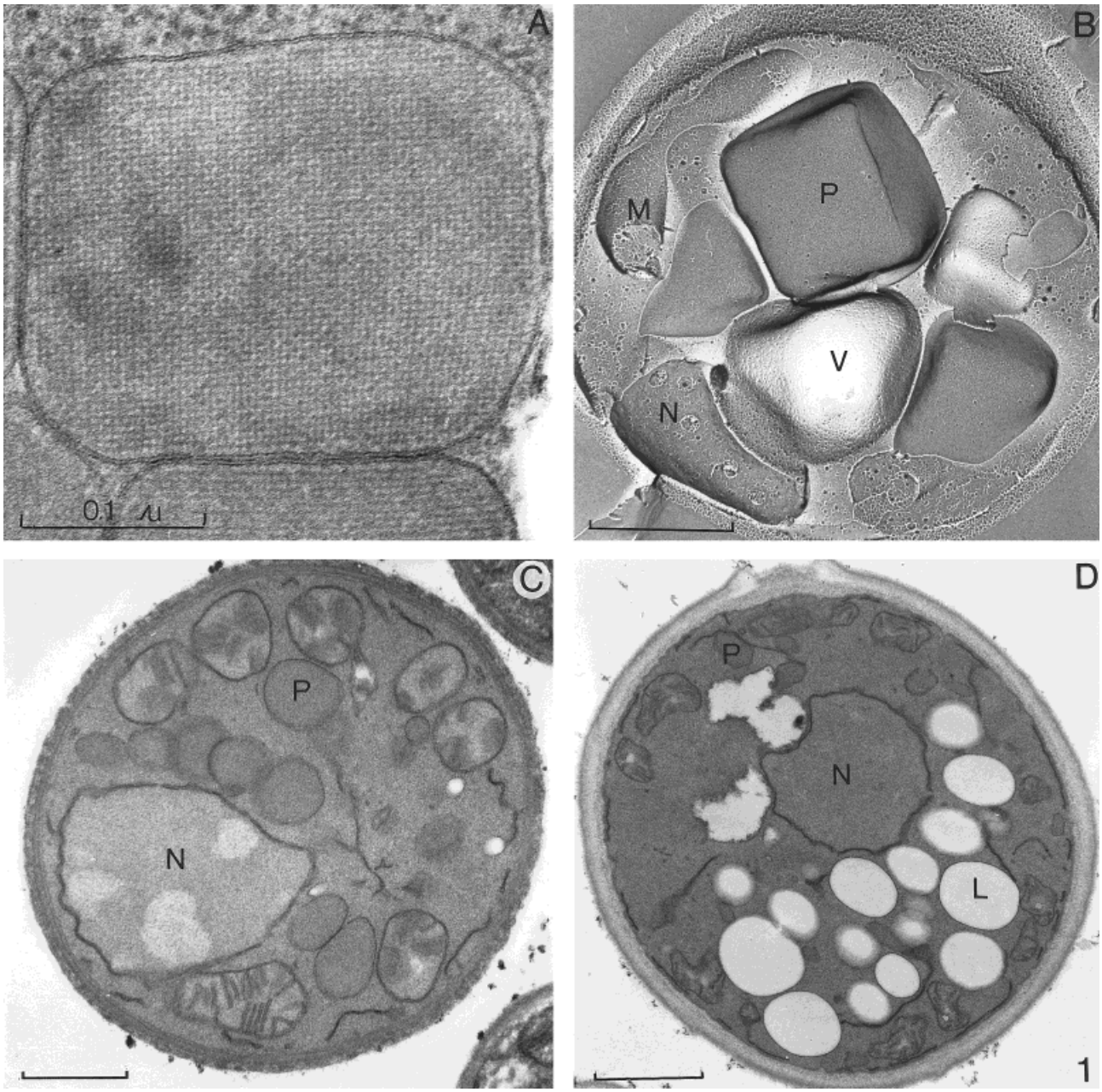

Fig. 1. Peroxisome proliferation in yeasts. **A:** *H. polymorpha* cell, grown in a methanol-limited chemostat, which contains cuboid peroxisomes due the crystalline nature of the matrix (glutaraldehyde/OsO_4) Bar = 0.1 μm. The crystalline matrix is composed of alcohol oxidase molecules. The cuboid shape of the organelles is also evident in freeze etch replica's of such cells (**B**). Other examples of peroxisome proliferation in yeasts are *Trichosporon cutaneum* grown on ethylamine as sole source of carbon and nitrogen (Veenhuis et al. 1986) (**C**) or oleic acid grown *S. cerevisiae* (Veenhuis et al., 1987). (**D**) For all figures electron micrographs are prepared from $KMnO_4$-fixed cells unless otherwise indicated. The bar represents 1 μm unless otherwise designated. Abbreviation: A–autophagosome; AV–autophagic vacuole; L–lipid droplet; M–mitochondrion; N–nucleus; P–peroxisome; V–vacuole.

proteins and the incorporation of lipids and membrane proteins, the size of the peroxisomes increases. However, growth of the organelles ceases when they have reached a certain size. At this stage only one, or infrequently few, new organelles are formed by fission from the mature one, which in turn start to grow during prolonged cultivation (Fig. 3). Apparently, these small organelles have "inherited" the capacity to grow from the mature parent, leaving this original organelle as an "enzyme bag," which is no longer capable of protein uptake (and most probably also multiplication) but remains physiologically active (Fig. 4). After multiple rounds of organellar growth and division, individual cells may contain over 20 peroxisomes. The observation that *H. polymorpha* peroxisomes are only temporarily competent for matrix protein import has been con-

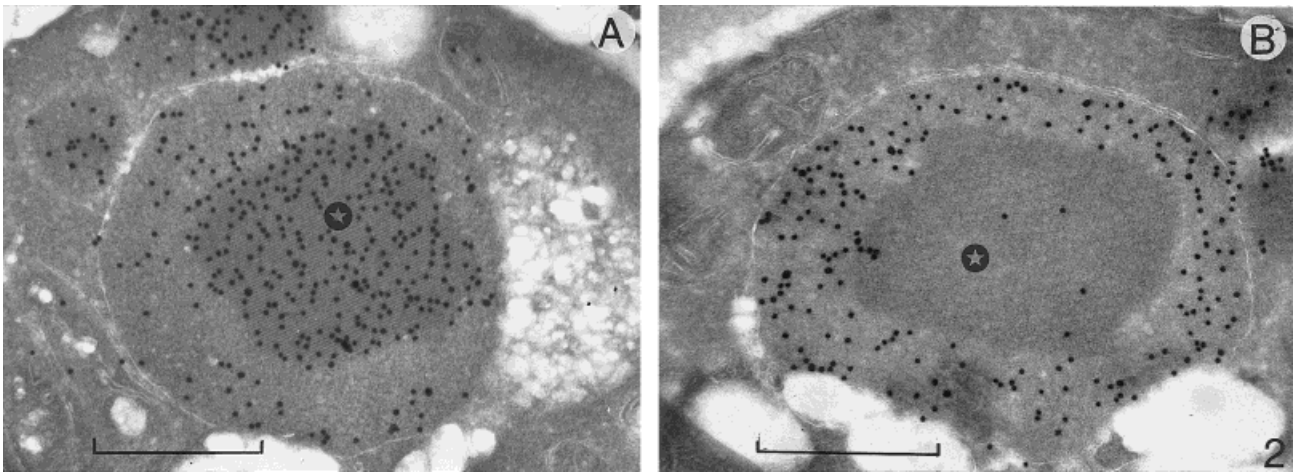

Fig. 2. Immunocytochemical localisation of alcohol oxidase (**A**) and catalase (**B**) in peroxisomes of *H. polymorpha* cells grown in batch culture on methanol. The location of catalase at the edge of the organelle is physiologically ideal to prevent leakage of hydrogen peroxide, produced by alcohol oxidase activity (aldehyde, uranyl acetate) Bar = 0.5 μm.

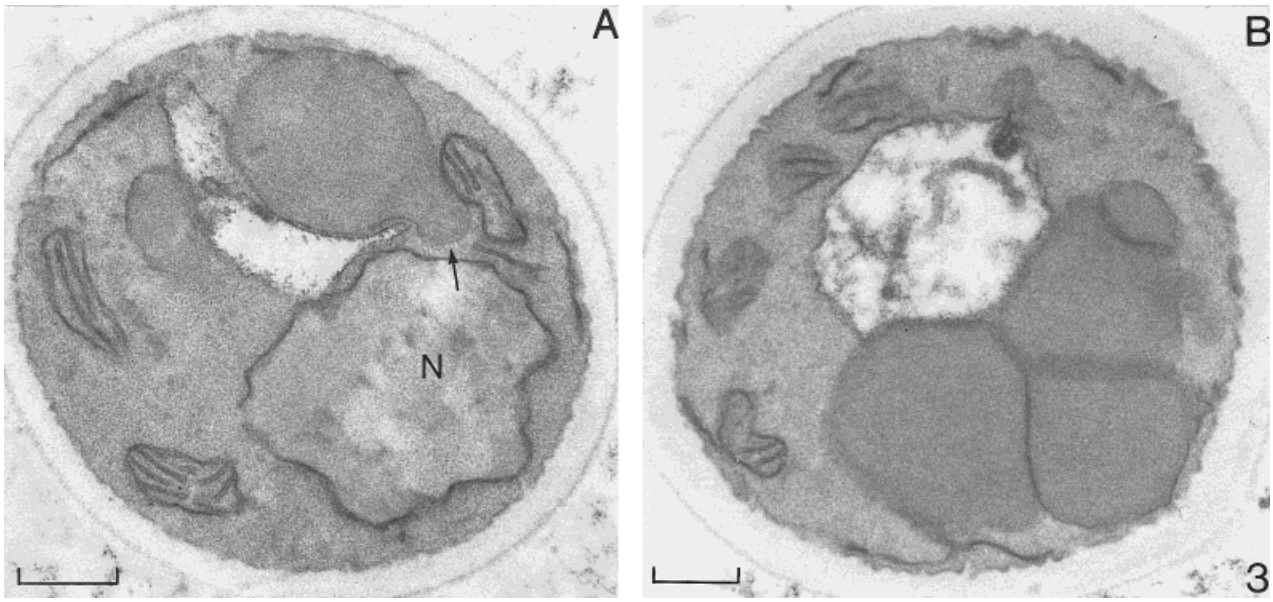

Fig. 3. Development of peroxisomes after a shift of *H. polymorha* cells from glucose to methanol. Initially one large organelle is formed that, upon maturation, divides by budding (**A**, arrow). At later stages, the cells contain several mature organelles together with few small import-competent ones.

firmed in a series of (immuno)cytochemical experiments that unequivocally demonstrated that newly induced enzymes are solely taken up by a few small, "immature" organelles (Veenhuis et al., 1989). In other yeast, slightly different proliferation mechanisms have been observed, but these most likely follow the same rule: peroxisomes are only temporarily capable to grow (demonstrated for *C. boidinii*, Waterham et al., 1992).

The difference between mature and immature peroxisomes is most likely related to variations in the biochemical properties of the organelles within one cell. In *Saccharomyces cerevisiae*, the oligomeric state of the peroxisomal membrane protein Pex11p could play a role in peroxisome maturation, because it is present as a monomer in small, newly formed organelles whereas it is predominantly dimeric in mature ones (Marshall et al., 1996).

When fully induced yeast cells are exposed to conditions in which peroxisomes are no longer required for growth (e.g., upon a shift to glucose containing media), the organelles are rapidly degraded by autophagy. Studies in *H. polymorpha* have suggested that mature organelles in particular are degraded leaving the small, import-competent ones unaffected (Fig. 4). The physiological advantage of this mechanism is immediately clear because it allows the cells to rapidly and

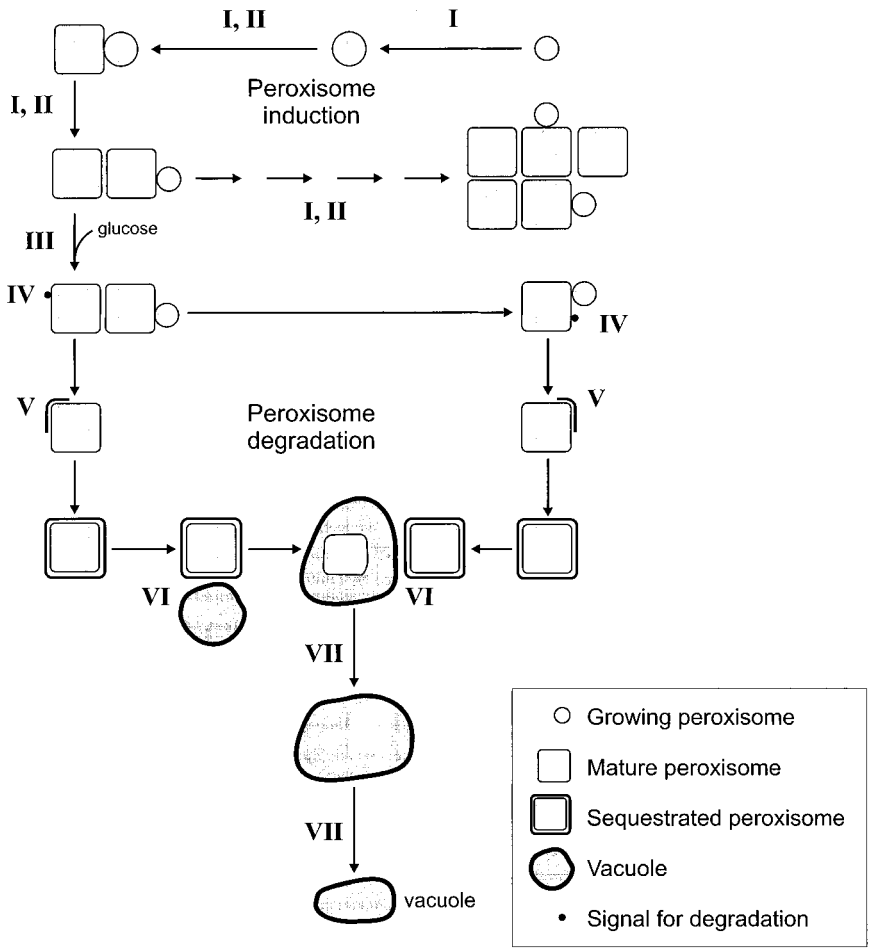

Fig. 4. Schematic representation of our current view on peroxisome biogenesis and degradation in *Hansenula polymorpha*. I, import of membrane and matrix components resulting in growth of the organelle; II, formation of a new organelle by division from a pre-existing one; III, induction of selective degradation by the addition of glucose; IV, putative tagging of the membrane of a mature peroxisome that is sensitive towards degradation; V, sequestering of the tagged organelle; VI, heterotypic fusion of the sequestered organelle with the vacuole; VII, degradation of the organelle in the vacuole.

optimally adapt to new growth environments that may require one or more new peroxisomal functions. Molecular studies on peroxisome degradation have now come within reach with the isolation of various yeast mutants that are defective in peroxisome degradation (*gsa, pag, pdd* mutants) and that will among others undoubtedly contribute to clues as to why few small, import-competent organelles escape degradation.

PEROXISOME BIOGENESIS

Genes Involved in Peroxisome Biogenesis (*PEX* Genes)

Molecular genetic approaches to identify proteins involved in peroxisome biogenesis (peroxins) became feasible in the early 1990s with the discovery that mutations that cause defects in peroxisome biogenesis or function (*pex* mutants; see Distel et al., 1996, for the unified nomenclature) are not lethal in yeast (Fig. 5). This property opened the way to use the power of yeast genetics to study peroxisome biogenesis (Lazarow, 1993). Yeast *pex* mutants have lost the capacity to grow on specific unusual carbon sources (e.g., fatty acids, methanol) that are metabolised by peroxisomal enzymes. This property facilitated cloning of *PEX* genes by transformation of mutants with genomic or cDNA libraries followed by selection of those transformants that have regained the capacity to grow on fatty acids and/or methanol. This approach has resulted in the identification of yet 23 different *PEX* genes from various yeasts (*H. polymorpha, S. cerevisiae, P. pastoris* and *Yarrowia lipolytica* (reviewed by Elgersma and Tabak, 1996; Hettema et al., 1999; Subramani, 1998). For most *PEX* genes orthologs are known from more than one yeast species (e.g., *PEX5*), which permits determination of the conserved regions in these genes. Using this information *PEX* orthologs from various other organisms have been identified, either by screening genome databases or by PCR using degenerate primers. In this way, for instance, *PEX5* orthologs were obtained from man (Dodt et al., 1995; Fransen et al., 1995; Wiemer et al., 1995), plant (Kragler et al., 1998; Wimmer et al., 1998) and trypanosomes (de Walque et al., 1999).

Most *PEX* gene products (termed *peroxins*) that have been identified so far are thought to play a role in matrix protein import. Other peroxins are implicated in peroxisomal membrane biogenesis, membrane protein insertion, or peroxisome proliferation.

Matrix Protein Import

Peroxisomal matrix proteins are encoded by nuclear genes and are synthesised in the cytosol on free ribo-

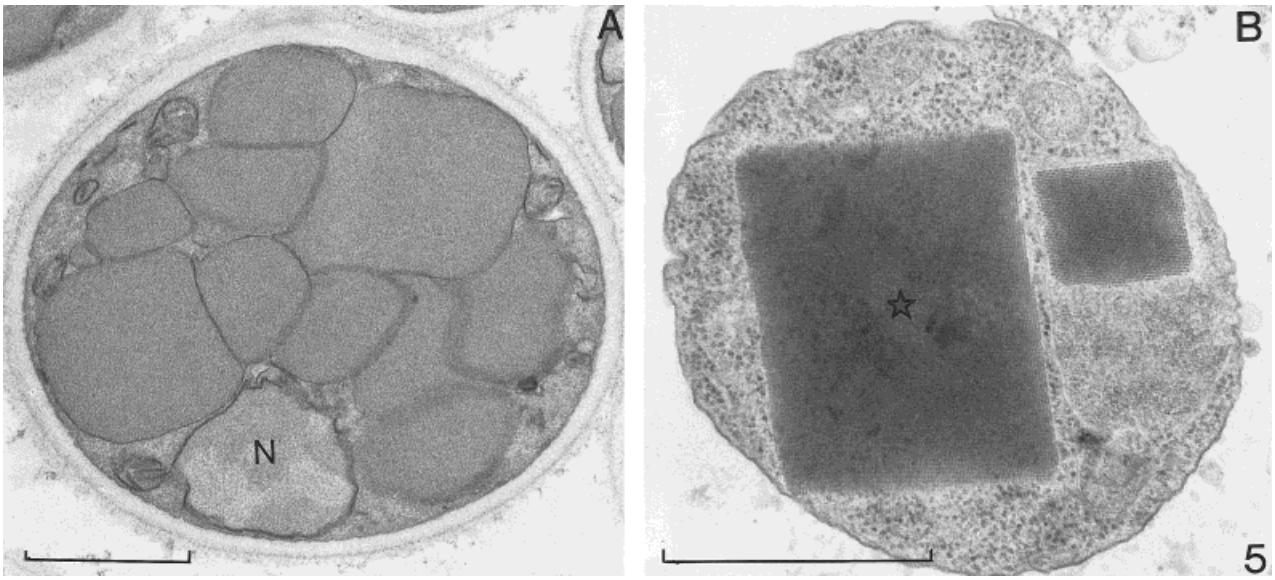

Fig. 5. Comparison of the morphology of WT and *pex* mutant cells of *H. polymorpha*, grown identically in a methanol-limited chemostat. The WT cells contains several peroxisomes (**A**) whereas in the mutant a large alcohol oxidase is observed in the cytosol, and also the nucleus, that lack a surrounding membrane (**B**; aldehyde, OsO_4)

somes (Lazarow and Fujiki, 1985). So far, two peroxisomal targeting signals have been characterised that are essential for sorting the protein to the proper organelle (De Hoop and AB, 1992; Rachubinski and Subramani, 1995; Subramani, 1998). The PTS1 signal is a tripeptide that is located at the extreme C-terminus of matrix proteins and is the most common peroxisomal targeting signal. The PTS1 consensus sequence is -S-K-L, but various (conserved) variants of this motif are allowed (Gould et al., 1989; Lametschwandtner et al., 1998). The PTS2 is present at the N-terminus of peroxisomal matrix proteins and consists of a nonapeptide with the consensus (R/K)-(L/V/I)-X_5-(H/Q)-(L/A).

PEX5 and *PEX7* encode the PTS1 and PTS2 receptors, respectively. The function of these genes was elucidated by analysis of the phenotype of yeast mutants in which either of the two genes was deleted. These studies revealed that in the absence of Pex5p, PTS1 protein import is fully impaired, whereas in strains that lack Pex7p, PTS2 protein import is defective. Because PTS2 import normally functions in yeast mutants lacking Pex5p and vice versa, the PTS1 and PTS2 protein import pathways can function independently in yeast.

A few matrix proteins are known that lack either a PTS1 or PTS2. Hence, besides the two identified signals, other peroxisomal targeting information must exist. Examples include *H. polymorpha* malate synthase (Bruinenberg et al., 1990) and *S. cerevisiae* catalase (Kragler et al., 1993). Possibly, these two proteins use the PTS1 import pathway, as import is impaired in mutants that lack the PTS1 receptor, Pex5p. In contrast, import of *S. cerevisiae* acyl-CoA oxidase, another protein that lacks a known PTS, is not hampered in either *pex5* or *pex7* mutants. Based on this finding, Zhang et al. (1993) proposed the existence of a third peroxisomal protein import pathway. This proposal is supported by a recent finding of Karpichev and Small (2000), who showed that in an *S. cerevisiae pex5-pex7* double mutant, the peroxisomal protein Dci1p, a putative enoyl-CoA hydratase, is still imported into the peroxisomal membrane remnants that are present in these cells.

PTS1 Protein Import

The initial data on the location of the PTS1-receptor, Pex5p, in different organisms were conflicting and varied with the organisms and/or the experimental approaches used. The reported locations varied from exclusively associated with the peroxisomal membrane, solely in the cytosol or the peroxisomal matrix, to a dual location in both the cytosol and the organellar matrix (Dodt et al., 1995; Elgersma et al., 1996; Gould et al., 1996; Szilard et al., 1995; Terlecky et al., 1995; Van der Klei et al., 1995; Wiemer et al., 1995). At present, it is generally accepted that the bulk of Pex5p is present in the cytosol, whereas a portion is associated with peroxisomes, bound to the peroxisomal membrane and present in the organellar matrix (Subramani, 1998). A major drawback in the biochemical approach to localise Pex5p was the strong sensitivity of this protein towards proteolytic degradation. In fractionation experiments vacuoles are readily damaged, resulting in the exposure of proteins of the cell homogenate to vacuolar proteases. As a result, especially soluble, cytosolic Pex5p is rapidly degraded, whereas peroxisome bound Pex5p is basically protected (Gould et al., 1996; Salomons et al., 2000; Van der Klei et al., 1998). This artefact does not occur in immunocytochemical experiments using intact, fixed cells; therefore, in this particular case the immunocytochemical

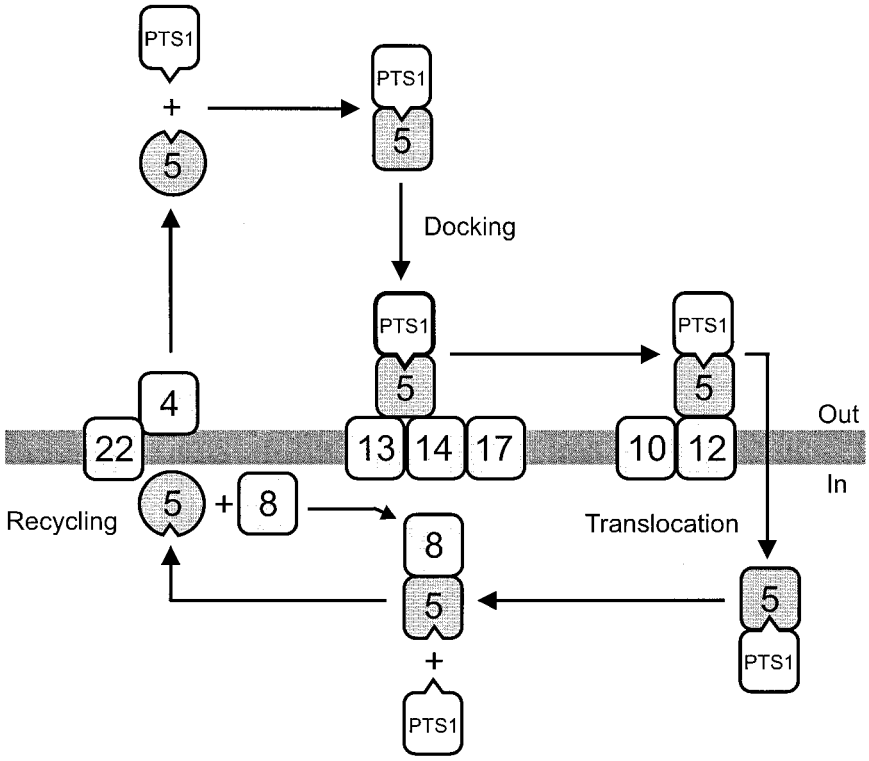

Fig. 6. The extended shuttling model of PTS1 matrix protein import in *H. polymorpha*. In the cytosol the PTS1 receptor, Pex5p, recognises the PTS1 of a newly synthesised matrix proteins. Possibly, this results in a conformational change (illustrated by the change of the round molecule into a cube) which leads to an increased affinity of Pex5p for the docking site at the peroxisomal membrane. This docking site is thought to contain the peroxins Pex13p, Pex14p and Pex17p. At a later stage Pex5p interacts with Pex12p, which binds to another membrane bound peroxin Pex10p. Then the receptor-cargo complex enters the peroxisome, where Pex8p may function in dissociating Pex5p from its cargo. Finally, Pex5p is exported and recycled to the cytosol, a process that involves Pex4p, which is anchored to the membrane via Pex22p.

data are probably more reliable than biochemical data on Pex5p location.

Based on both cell fractionation studies and immunocytochemistry, we showed that in *H. polymorpha* Pex5p is present in the cytosol and in the peroxisomal matrix. In WT *H. polymorpha*, the amount of Pex5p associated with the peroxisomal membrane is invariably low and below the limit of detection (Van der Klei et al., 1995, 1998). These observations are the rationale of our current model that predicts that *H. polymorpha* Pex5p functions as a cycling receptor between the cytosol and the peroxisomal matrix (Van der Klei and Veenhuis, 1996; Fig.6). According to this model, the first step in matrix protein import is the recruitment of a newly synthesised PTS1 protein by Pex5p in the cytosol. Subsequently, the Pex5p-cargo complex docks to the organelle and then enters the matrix where the PTS1 protein dissociates from its receptor. Finally, Pex5p is recycled to the cytosol where it can mediate another round of import.

Two-hybrid analysis and in vitro protein binding assays have shown that the tetratricopeptide repeats (TPR) in the C-terminal half of Pex5p bind the PTS1 (Brocard et al., 1994; Fransen et al., 1995; Szilard and Rachubinski, 2000; Terlecky et al., 1995). The eight residues that precede the carboxyl terminal PTS1 tripeptide influence binding and probably modulate the strength of the interaction (Lametschwandtner et al., 1998). The N-terminal half of Pex5p has been implicated to interact with Pex13p (Urquhart et al., 2000) and Pex14p (Schliebs et al., 1999).

Upon binding a PTS1, the receptor-cargo complex interacts at a putative docking site on the membrane en route to the peroxisomal matrix. This model implies that the affinity of Pex5p for the docking site at the peroxisomal membrane increases upon binding of a PTS1 and explains why only a minute portion of native Pex5p is associated with the peroxisomal membrane. Peroxins that have been suggested to participate in Pex5p/cargo docking are Pex13p (Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996), Pex14p (Albertini et al., 1997; Brocard et al., 1997; Komori et al., 1997), and Pex17p (Huhse et al., 1998; for reviews see Erdmann et al., 1997; Subramani, 1998). This function for Pex13p and Pex14p was related to the fact that these peroxins are constituents of the peroxisomal membrane and contain cytosolically exposed domains that can physically interact with Pex5p (based on two-hybrid analysis). Moreover, deletion of either of the genes encoding Pex13p or Pex14p results in a general defect in matrix protein import. Two-hybrid analysis revealed that Pex13p and Pex14p also interact with each other, suggesting that these proteins may be part of a docking complex that also contains Pex17p. Pex17p is a peroxisomal membrane protein involved in matrix protein import that interacts with Pex14p, but not directly with Pex5p and Pex13p (Huhse et al., 1998; Fig. 6).

Two-hybrid analysis and co-immune precipitation experiments suggested that Pex5p interacts with Pex7p, Pex8p, Pex12p, Pex13p, Pex14p, and Pex17p. Using the common *S. cerevisiae* host strains for two-hybrid analysis, it is not possible to discriminate between direct and indirect interactions, because the yeast cells express numerous other genes except for the two that are actually studied. For instance, the inter-

actions Pex5p-Pex7p and Pex5p-Pex17p observed in two-hybrid screens were shown to be indirect and dependent on the expression of *PEX14* by the host strain (Girzalsky et al., 1999; Huhse et al., 1998). Hence, it is also essential to analyse putative peroxin interactions biochemically using purified components in order to discriminate between direct and indirect interactions.

An elegant example of such analyses was recently published by Schliebs et al. (1999) who studied Pex5p/Pex14p interactions in vitro. Two-hybrid analysis had revealed that the first 58 N-terminal amino acids of *S. cerevisiae* Pex14p were involved in Pex5p binding. Schliebs et al. (1999) isolated the corresponding N-terminal fragment of human Pex14p to study its interactions with purified human Pex5p. Kinetic analysis by surface plasmon resonance spectroscopy revealed that the N-terminal Pex14p fragment very strongly binds Pex5p (equilibrium binding constant of approximately 1 nM). Surprisingly, the strong binding was observed in the absence of PTS1 cargo molecules. The observation that only a minute portion of Pex5p is bound to the peroxisomal membrane suggests that the affinity of Pex5p for Pex14p is much lower in vivo. Possibly other proteins (peroxins, PTS1 cargo proteins), other domains of Pex14p, or the recently reported phosphorylation of Pex14p (Komori et al., 1999) may influence the strength of Pex5p-Pex14p binding.

Analysis of in vitro formed Pex5p-Pex14p protein complexes revealed that one Pex5p molecule contains 5–7 binding sites for the N-terminal Pex14p fragment (Schliebs et al., 1999). Most likely, the different binding sites are used one by one, resulting in a cascade of binding and release during the protein import process.

Direct binding between full-length Pex5p and Pex14p molecules has also been demonstrated using in vitro overlay bindings assays (human Pex5p and Pex14p, Fransen et al., 1998; *P. pastoris* Pex5p and Pex14p, Urquhart et al., 2000). In the studies with *P. pastoris* Pex5p, a slight increase in Pex5p binding was observed when Pex5p was preloaded with a PTS1 peptide.

Although it is generally accepted that Pex14p plays a central role in peroxisomal matrix protein import, *H. polymorpha* Pex14p is not essential for this process. We demonstrated that the PTS1 import defect in a *H. polymorpha PEX14* null mutant can be largely restored by overproduction of Pex5p (Salomons et al., 2000). Under these conditions, a major amount of Pex5p accumulated at the outer surface of the peroxisomal membrane, a phenomenon that is never observed in *H. polymorpha* WT cells, also not when Pex5p is overproduced (Van der Klei et al., 1995, 1998). These data suggest, therefore, that Pex14p is not essential for the initial Pex5p docking, but may function at a later stage, where it is important for the efficiency of the import process. These experiments also showed that not only the PTS1 is the prime factor that determines import but that additional information must exist in the PTS1 import machinery. This was among others clear from the observation that AO and DHAS import was promoted in Δ*pex14* cells overproducing Pex5p, whereas catalase—another PTS1 protein—remained mislocated in the cytosol.

Compared to the interaction of Pex5p-Pex14p, the interaction of the Pex5p with Pex13p seems to be much weaker. Using in vitro overlay binding assays Fransen et al. (1998) were unable to detect direct physical interaction between full-length human Pex5p and Pex13p molecules. However, using a similar technique, *P. pastoris* Pex5p was shown to bind a fragment consisting of the SH3 domain of *P. pastoris* Pex13p (Urquhart et al., 2000). The amount of Pex5p bound to the Pex13p fragment was 20–40 times lower compared to the amount of Pex5p that bound to Pex14p under similar conditions (Urquhart et al., 2000). Hence, in the experiments by Fransen et al. (1998) the interaction may have been below the level of detection. Alternatively, in vitro binding of Pex5p to Pex13p is prevented or reduced when full-length Pex13p is used. Indeed, Urquhart et al. (2000) showed that Pex5p most effectively bound to a fragment containing just the SH3 domain, whereas the interaction was weakened when larger Pex13p fragments were used.

Although Pex13p, Pex14p, and Pex17p are proposed to form the Pex5p docking site at the peroxisomal membrane, the molecular functions of these proteins are still speculative. Based on the Pex5p binding properties, Pex14p seems to be the most likely candidate for recruiting Pex5p at the peroxisomal membrane (Schliebs et al., 1999; Urquhart et al., 2000). However, our recent findings (Salomons et al., 2000), which suggest that Pex14p is dispensable for Pex5p docking, are inconsistent with this role for Pex14p. Furthermore, Urquhart et al. (2000) recently suggested an alternative role for Pex13p in a later stage of the process that occurs after docking. Further studies are, therefore, necessary to determine which, possibly novel, peroxins are involved in initial Pex5p docking.

Human Pex5p was recently shown to interact with Pex12p (Chang et al., 1999; Fig. 6). Pex12p is an integral peroxisomal membrane protein that contains a C-terminal zinc-binding domain, which is exposed to the cytosol and responsible for Pex5p binding. The same domain in Pex12p also binds Pex10p, another zinc-binding peroxisomal membrane protein (Chang et al., 1999). Both *pex10* and *pex12* mutants are specifically defective in peroxisomal matrix protein import, indicating an important role of these genes in matrix protein import. Since the association of Pex5p with the peroxisomal membrane is not reduced in human cell lines affected in *PEX10* or *PEX12*, Chang et al. (1999) speculated that both genes function at a stage that occurs after the docking event (Fig. 6).

The presence of a portion of Pex5p in the peroxisomal matrix of *H. polymorpha*, led us to propose the so-called extended shuttle model, which predicts that Pex5p is translocated together with its PTS1 cargo protein into the organellar matrix (Van der Klei and Veenhuis, 1996). Pex8p is a peroxisomal matrix protein that is associated with the inner surface of the peroxisomal membrane and essential for matrix protein import. This location together with the finding it interacts with Pex5p suggests that Pex8p functions at an intraorganellar stage of the PTS1 protein import cascade. A characteristic feature of the Pex8p's known so far is that they contain a PTS1 (Liu et al., 1995; Rehling et al., 2000; Waterham et al., 1994). The obvious function of this signal is to target newly synthesised Pex8p to peroxisomes. However, Waterham et al. (1994) showed that *H. polymorpha* Pex8p, from which the PTS1 is

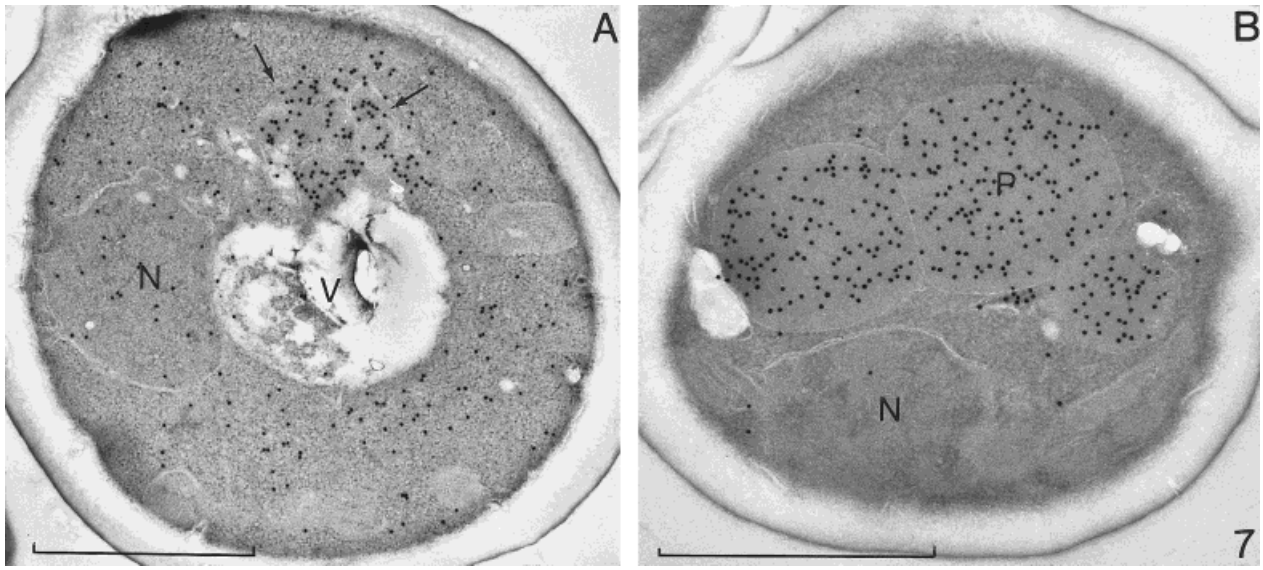

Fig. 7. Immunocytochemical demonstration of alcohol oxidase in small peroxisomes (arrows) and cytosol of Δ*pex 4* cells (**A**; aldehyde, uranyl acetate). After overproduction of Pex5p in the Δ*pex 4* strain most of the alcohol oxidase protein accumulates in enlarged peroxisomes (**B**; aldehyde, uranyl acetate).

removed, is normally imported into peroxisomes and fully functional. In addition, truncated *S. cerevisiae* Pex8p that lacks the PTS1 is still capable to complement the matrix protein import defect in a *PEX8* deletion strain (Δ*pex8*). Surprisingly, the truncated form of *S. cerevisiae* Pex8p still interacts with Pex5p (Rehling et al., 2000). The significance of this interaction, which is expected to occur in the peroxisomal matrix, and the molecular function of Pex8p, is a challenging aspect in current studies on Pex5p-mediated matrix protein import; one of the possible options is that Pex8p functions in the release of the cargo protein from the PTS1 receptor (Fig. 6).

A peroxin that is proposed to be involved in a late stage of Pex5p-dependent protein import is the ubiquitin-conjugating enzyme Pex4p (Crane et al., 1994; Van der Klei et al., 1998; Wiebel and Kunau, 1992). Pex4p is associated to the peroxisomal membrane by binding to the integral peroxisomal membrane protein Pex22p and faces the cytosol (Koller et al., 1999; Fig. 6). *Pex4* mutants share a general block in PTS1 protein import. *S. cerevisiae* and *P. pastoris pex4* mutants are also defective in import of the PTS2 protein thiolase, while the PTS2 protein amine oxidase is still normally imported in peroxisomes of *H. polymorpha pex4* mutant cells. Whether these differences are related to specific differences in the PTS2 marker proteins used (thiolase vs. amine oxidase) remains to be established.

Like in *H. polymorpha* Δ*pex14*, overproduction of Pex5p also suppresses the PTS1 import defect in *H. polymorpha* Δ*pex4* cells (Fig. 7). Surprisingly, in Δ*pex4* cells overproducing Pex5p this protein accumulated at the inner surface of the peroxisomal membrane (Van der Klei et al., 1998), thus exact the opposite location as in Δ*pex14*cells overproducing Pex5p (Salomons et al., 2000). Consistent with the extended Pex5p recycling model (Dodt and Gould, 1996; Van der Klei and Veenhuis, 1996), this observation implies that export and recycling of Pex5p to the cytosol are blocked in the absence of Pex4p. As a result, Pex5p accumulates inside peroxisomes whereas the cytosolic Pex5p pool is depleted. By overproduction of Pex5p, the cytosolic pool is, however, replenished, explaining the almost complete restoration of the PTS1 import defect in *pex4* null mutants upon overexpression of *PEX5*. Interestingly, also in this case import of AO and DHAS was strongly stimulated by Pex5p overproduction, whereas catalase remained cytosolic. Since Pex4p is a protein in the family of ubiquitin conjugating enzymes, Pex5p recycling probably involves the modification of a yet unknown protein by ubiquitination.

Alternative pathways have been proposed that do not include import of Pex5p into the peroxisomal matrix (Erdmann et al., 1997; Hettema et al., 1999; Olsen, 1998; Subramani, 1998; Urquhart et al., 2000; Waterham and Cregg, 1997). In this model, the Pex5p/PTS1 cargo complex docks at the peroxisomal membrane, followed by delivery of the cargo to a translocation site and recycling of the receptor to the cytosol. The fundamental difference between both models is that in the extended shuttle model, the matrix-located Pex5p is considered to represent a functional intermediate in the import process whereas in the alternative model matrix-located Pex5p molecules are considered to be non-functional, "dead-end" molecules that accidentally entered the matrix. The experimental data available so far do not yet confirm which of the two import models is correct.

Since in *Y. lipolytica* Pex5p is exclusively located in the peroxisomal matrix, Szilard et al. (1995) suggested another function for Pex5p in that it pulls PTS1 proteins across the membrane into the matrix. Since in other organisms invariably only a minor portion of Pex5p is found in the peroxisomal matrix, this "pulling" model does not seem to reflect a common Pex5p function.

PTS2 Protein Import

Only a limited number of peroxisomal matrix proteins contain a PTS2. Examples are thiolase that is involved in β-oxidation in mammals and yeast (Glover et al., 1994a; Swinkels et al., 1991) and amine oxidase present in *H. polymorpha*, grown in media containing primary amines as sole nitrogen source (Faber et al., 1995). The intracellular location of the PTS2 receptor, Pex7p, has been described for several organisms, but is still controversial. For example, in *S. cerevisiae* Pex7p tagged with three repeats of haemagglutinin at the carboxyterminus (Pex7p-HA_3) was exclusively found in the peroxisomal matrix when expressed at wild-type levels. However, upon overexpression Pex7p-HA_3 also accumulated in the cytosol (Zhang and Lazarow, 1996). Conversely, an N-terminally Myc-tagged version of *Sc*Pex7p was predominantly cytosolic (Marzioch et al., 1994). Similarly, mammalian Pex7p, tagged with a Myc-epitope at the N-terminus, was exclusively found in the cytosol (Braverman et al., 1997). On the other hand, untagged *Pp*Pex7p showed a dual location both in the cytosol and peroxisomal matrix (Elgersma et al., 1998). The last data may be most reliable, because the endogenous native protein was localised without any tags that might possibly influence the final location. Like Pex5p, Pex7p interacts with Pex13p (Girzalsky et al., 1999) and Pex14p (Albertini et al., 1997). Hence, it is tempting to speculate that Pex7p follows a similar pathway as Pex5p to guide peroxisomal matrix proteins to the correct subcellular location.

*Sc*Pex7p also interacts with two cytosolic peroxins, namely Pex18p and Pex21p (Purdue et al., 1998). Cells lacking either of these peroxins fail to import PTS2 proteins. Because the haemaggluttinin tagged Pex7p (Pex7p-HA_3) was located in the cytosol of cells lacking Pex18p and Pex21p, Purdue and co-workers (1998) argued that both proteins are key components in targeting Pex7p to peroxisomes.

Peroxisomal Matrix Protein Assembly

At present, little is known about the mechanisms involved in the assembly of peroxisomal matrix proteins. In fact, even the subcellular site at which matrix proteins are assembled and activated is still a matter of debate.

It has been shown that some matrix enzymes are assembled into oligomers in the cytosol before being imported into the peroxisomal matrix (McNew and Goodman, 1996). For instance, in *S. cerevisiae* thiolase subunits lacking the PTS2 can be "piggy-back-imported" with PTS2-containing subunits after oligomerisation in the cytosol (Glover et al., 1994b). Similarly, import of chloramphenicol transferase subunits lacking a PTS1 occurred in *S. cerevisiae* and mammalian cells when subunits containing a PTS1 were synthesised as well (McNew and Goodman, 1994). In plant isocitrate lyase subunits lacking a PTS were shown to be "piggybacked" to glyoxysomes (Lee et al., 1997). In addition, evidence has been obtained that the peroxisomal import machinery can accommodate complex, folded proteins like albumin to which PTS1 containing peptides are cross-linked. In fact, even 9-nm gold particles coated with PTS1-peptides were shown to be taken up by peroxisomes upon microinjection into mammalian cells (Walton et al., 1995). However, although these experiments indicate that folded, oligomeric proteins can be transported across the peroxisomal membrane, this does not necessarily mean that this is the normal scenario of matrix protein import in wild type cells. In fact, for a few proteins, e.g., AO in methylotrophic yeast, evidence has been presented that indicates that oligomerisation and activation occur after translocation in the peroxisomal matrix (Evers et al., 1996; Goodman et al., 1984; Waterham et al. 1997).

Since peroxisomal matrix proteins do not necessarily have to be unfolded during translocation, the requirement of the function of molecular chaperones of the Hsp70 protein family in this process is also puzzling. For import of precursor proteins into mitochondria or the endoplasmic reticulum (ER), cytosolic Hsp70s have been implicated to keep the precursors in an unfolded, import competent conformation. In import experiments using semi-permeabilised mammalian cells, the addition of Hsp70 antibodies inhibited peroxisomal protein import (Walton et al., 1994). Similarly, addition of antibodies against Hsp70 or Hsp90 to a plant in vitro import assay inhibited peroxisomal protein import (Crookes and Olsen, 1998). The precise role of these cytosolic chaperones, if any, in matrix protein import remains to be elucidated.

In yeast *pex* mutants that are defective in matrix protein import, the assembly of peroxisomal matrix proteins is generally not affected, but normally proceeds in the cytosol (Fig. 5B). However, a few mutants have been described in which both the import and the assembly of specific enzymes is affected. These include *Y. lipolytica pex20*, a *C. boidinii* mutant lacking the peroxisomal membrane protein Pmp47 and a *H. polymorpha* pyruvate carboxylase (PYC) deficient strain. *Y. lipolytica pex20* is selectively blocked in the import and assembly of thiolase. Pex20p is a cytosolic protein that forms a heteromeric complex with newly synthesised thiolase subunits. Possibly, Pex20p acts as a cytosolic chaperone assisting oligomerisation of thiolase into dimers that normally may occur prior to its import into the peroxisome (Titorenko et al., 1998).

In *C. boidinii*, deletion of the gene encoding the peroxisomal membrane protein PMP47 (Δ*pmp47*) resulted in a specific defect in import of the PTS1 protein DHAS and the formation of inactive DHAS protein aggregates in the cytosol (Sakai et al., 1996). Interestingly, when the formation of peroxisomes was prevented (by introducing a *PEX* deletion in this strain), the cytosolic DHAS protein was activated again. These findings, therefore, suggest that Pmp47 is not directly involved in DHAS assembly. Possibly, a peroxisomal factor that is spatially separated from cytosolic DHAS in Δ*pmp47*, but not in a *pex*-Δ*pmp47* double mutant, is necessary for proper DHAS assembly.

In *H. polymorpha*, several lines of evidence suggest that assembly of the peroxisomal matrix protein AO into an active homo-octameric flavo-enzyme is not a spontaneous process in vivo, but involves helper proteins (chaperones). In order to identify genes involved in AO assembly, we isolated a collection of *H. polymorpha* mutants that were affected in AO assembly/activation. One of these mutants was characterised by the accumulation of inactive, monomeric AO protein in the

cytosol, but in addition required aspartate or glutamate for growth. By functional complementation of the AO assembly defect, the gene encoding the enzyme pyruvate carboxylase (*PYC*) was cloned. Although the absence of the PYC enzyme activity could be overcome by the addition of aspartate or glutamate to the growth medium, the inability to assemble AO and hence to grow on methanol could only be restored by re-introduction of the *PYC* gene. Therefore, these data imply that, apart from its known metabolic activity, pyruvate carboxylase fulfills a second function that is required for AO assembly. Identical observations were made in *P. pastoris* (Van Dijk et al., unpublished results).

Involvement of Preperoxisomes in Matrix Protein Import

Upon their discovery, the initial morphological data led to the hypothesis that peroxisomes developed by budding from the endoplasmic reticulum (De Duve and Baudhuin, 1966). However, the finding that peroxisomal proteins are synthesised in the cytosol on free polysomes at their mature size followed by post-translational import into peroxisomes, changed this concept into a novel model, in which new peroxisomes are proposed to derive by budding from pre-existing peroxisomes (Lazarow and Fujiki, 1985).

Several reports have provided evidence that the newly formed, small peroxisomes that are competent to incorporate matrix proteins, have a lower density compared to that of the bulk of the peroxisomes in a cell, which typically migrate to high densities upon density gradient centrifugation. Heinemann and Just (1992) showed in a series of pulse chase experiments on rat hepatocytes that newly synthesised peroxisomal acyl-CoA oxidase is first imported into organelles of an intermediate density (1.16–1.17 g/cm^3), whereas in a later stage the enzyme is found in peroxisomes of higher density (1.22–1.23 g/cm^3). Similarly, biochemical analysis of shortly induced *S. cerevisiae* cells suggested that during induction peroxisomes of high density (1.23 g/cm^3) arise from low-density organelles (1.15 g/cm^3; Erdmann and Blobel, 1995). In addition, in *H. polymorpha* small peroxisomal structures have been observed that are of a lower density than mature organelles. For example, overproduction of the peroxisomal membrane protein Pex3p resulted in numerous, small import competent peroxisomes with a lower density (1.18 g/cm^3) compared to normal peroxisomes (1.23 g/cm^3; Baerends et al., 1997).

Further evidence that matrix protein import might involve low-density vesicles or pre-peroxisomes was recently provided by Titorenko et al. (2000), who identified six distinct peroxisomal subforms, designated P1–P6, in *Y. lipolytica*. These subpopulations differed in density from 1.21 g/cm^3 for mature peroxisomes (P6) to 1.18 g/cm^3 (P5), 1.14 g/cm^3 (P3+P4), 1.11 g/cm^3 (P1), and 1.09 g/cm^3 (P2) for the different subpopulations. The subpopulations did not only differ in density, but were characterised by differences in protein composition. Pulse-chase analysis revealed that the bulk of newly synthesised malate synthase was initially found in the cytosol. Subsequently, the protein was first chased to low density structures (P1 and P2), after which it moved to the mature organelles (P6) via the other subpopulations (P3-P5). This implies that the development of peroxisomes involves the conversion of small, low-density preperoxisomal vesicles eventually to a high-density mature peroxisome. This mode of peroxisome formation includes import of various matrix proteins into distinct classes of preperoxisomes. For example, isocitrate lyase was detected in P4, P5, and P6 populations, but was absent in earlier subforms. Moreover, using an in vitro fusion assay Titorenko et al. (2000) showed that the P1 and P2 subforms could fuse into the larger and denser peroxisomal subform P3. Pex1p and Pex6p, members of the AAA-protein family (Confalonieri and Duguet, 1995), were implicated to play a role in this process. This phenomenon observed in the yeast *Y. lipolytica* offers novel insights into peroxisome biogenesis. However, the presence of various peroxisomal subforms containing an assortment of peroxisomal proteins was not yet detected in other organisms. Hence, whether the observations made in *Y. lipolytica* represent a general mechanism of peroxisome biogenesis in eukaryotes remains to be established.

Biogenesis of the Peroxisomal Membrane

Besides matrix protein import, the proliferation of peroxisomes also requires recruitment of phospholipids and insertion of membrane proteins (for review, see Baerends et al., 2000b; Fig. 8). It has not been established yet how phospholipids are incorporated into the peroxisomal membrane. Recently, several lines of indirect evidence suggested the involvement of the ER (reviewed by Titorenko and Rachubinski, 1998). For example, brefeldin A, a fungal toxin that interferes with coated vesicle formation, inhibited peroxisome biogenesis in *H. polymorpha* and resulted in the accumulation of peroxisomal membrane and matrix proteins at the ER (Salomons et al., 1997).

Fusion of the putative ER-derived vesicles with the peroxisome has been suggested to be mediated by two peroxins, Pex1p (Erdmann et al., 1991) and Pex6p (Spong and Subramani, 1993; Voorn-Brouwer et al., 1993), that show homology to NSF and Sec18p, proteins involved in other membrane fusion processes (Eakle et al., 1988; Wilson et al., 1989). Moreover, the involvement of Pex1p and Pex6p in vesicle fusion has recently been demonstrated in vitro using pre-peroxisomal vesicles isolated from *Y. lipolityca* (Titorenko et al., 2000). A possible functional connection of Pex1p and Pex6p with the ER was earlier suggested based on the finding that *Y. lipolytica pex1* and *pex6* mutants are both defective in peroxisome biogenesis and in exit of some secretory proteins from the ER (Titorenko and Rachubinski, 1998).

The sorting and insertion of peroxisomal membrane proteins into the membrane require another type of signal than the PTS1 and PTS2 of matrix proteins and a different insertion machinery. Evidence for this has become apparent in yeast cells defective in PTS1 and PTS2 import. In these cells, peroxisomal membrane structures (ghosts) were observed in which peroxisomal membrane proteins are normally inserted (Purdue and Lazarow, 1995; Subramani, 1993; Veenhuis et al., 1996). Furthermore, some membrane proteins contain sequences, called mPTSs, which are essential and sufficient for the targeting proteins to the peroxisomal membrane. In *C. boidinii*, Pmp47p a mPTS was found

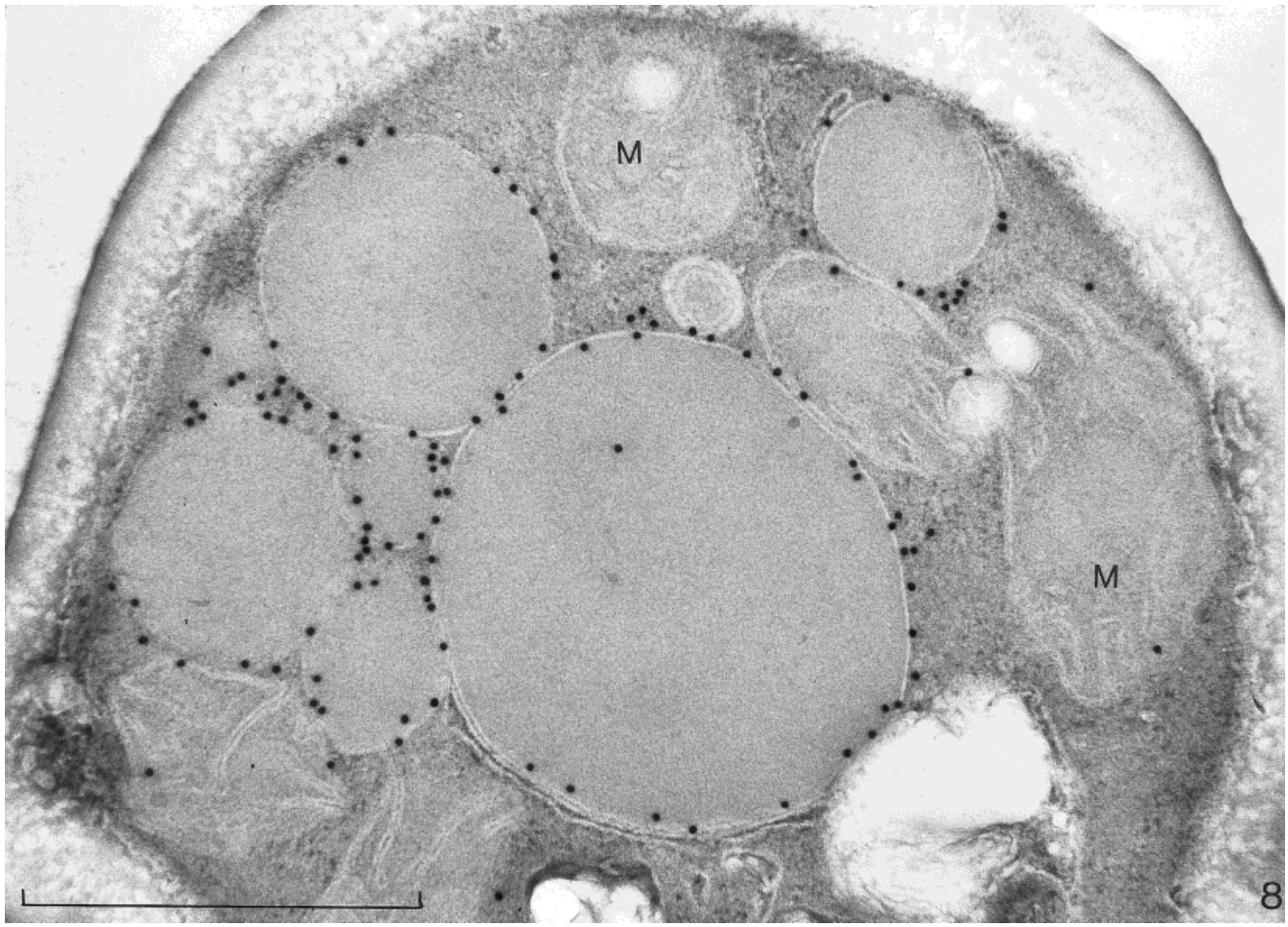

Fig. 8. Immunocytochemical demonstration of Pex3p in the membrane of *H. polymorpha* peroxisomes (aldehyde, uranyl acetate).

to be located within a 20 amino acid loop between two transmembrane domains (Dyer et al., 1996). Another membrane protein, Pex3p (Fig. 8) contains targeting information within the N-terminus. The first 40–45 aminoterminal region of *P. pastoris* Pex3p is sufficient to sort a reporter protein to peroxisomes (Wiemer et al., 1996). Recently, Baerends et al. (2000a) showed that a conserved stretch of positively charged amino acids (amino acids 11–15) in the N-terminus of *H. polymorpha* Pex3p is involved in the incorporation of the protein in the peroxisomal membrane. However, a reporter protein fused to the first 16 aminoterminal amino acids of *H. polymorpha* Pex3p was predominantly sorted to the ER (Baerends et al., 1996). These findings suggest a possible role of the ER in sorting of membrane proteins to the peroxisomal membrane. Moreover, in *Y. lipolytica* two peroxisomal membrane proteins, Pex2p and Pex16p, are first targeted to the ER and subsequently transported to peroxisomes (Titorenko and Rachubinski, 1998).

So far, at least four peroxins, Pex3p (Baerends et al., 1996; Wiemer et al., 1996), Pex16p (South and Gould, 1999), Pex17p (Snyder et al., 1999a), and Pex19p (Götte et al., 1998; Snyder et al., 1999b; Hettema et al., 2000; Sacksteder et al., 2000) are thought to be involved either in the formation of peroxisomal membranes or in import of membrane proteins. However, the dynamics of these mechanisms and the function of the peroxins therein remain speculative.

PEROXISOME DEGRADATION

In eukaryotic cells, whole organelles can be degraded inside the vacuole/lysosome by a process called autophagy. This process involves the uptake of organelles in the hydrolytic compartment, followed by degradation of their components (proteins, lipids) by vacuolar hydrolases (Fig. 4). Peroxisomes can be degraded either nonselectively, together with other cytoplasmic components, or selectively in a process where solely peroxisomes are degraded.

In yeast, peroxisome degradation has been observed under various experimental conditions. During nonselective autophagy, major portions of the cytoplasm, including peroxisomes, are degraded. This process is, for instance, induced under nitrogen starvation conditions (Takeshige et al., 1992). A more selective mode of peroxisome degradation occurs when yeast cells are shifted to a carbon source, which renders the function

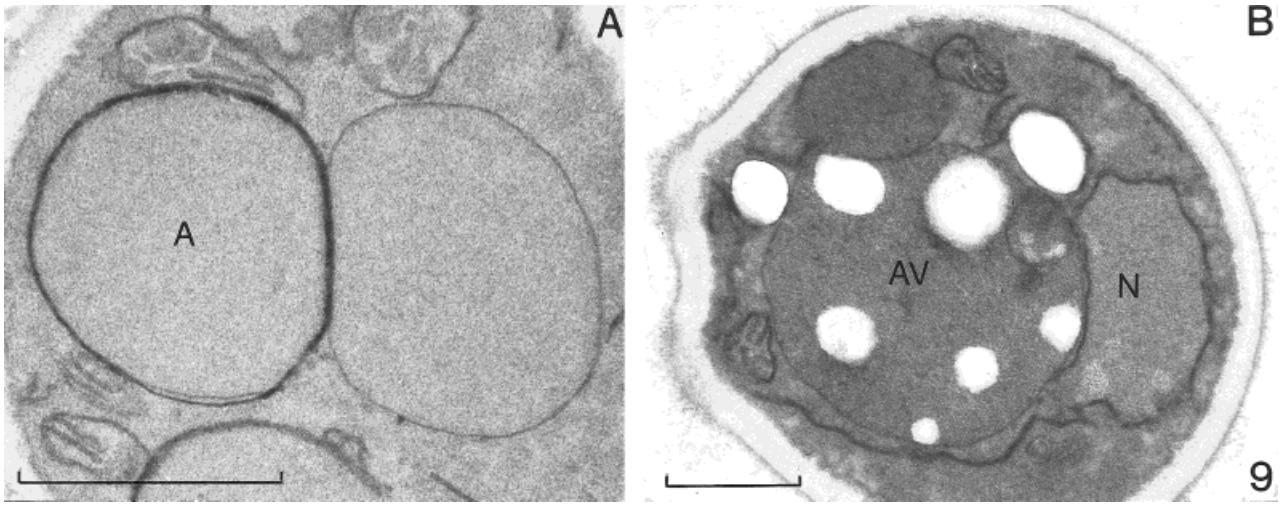

Fig. 9. Two stages of selective peroxisome degradation in *H. polymorpha* cells, shifted from methanol into glucose-containing media. **A:** The autophagosome, formed after sequestration of an individual peroxisome. **B:** Degeneration of the organelle is visualised in the autophagic vacuole (AV). Note that the small organelle is not affected. Bar = 0.5 μm

of the organelles redundant for growth. The first reports of carbon source induced peroxisome degradation include glucose- or ethanol-induced turnover in the methylotrophic yeasts *C. boidinii* (Bormann and Sahm, 1978; Hill et al., 1985) and *H. polymorpha* (Veenhuis et al., 1978; 1983; Fig. 9). Methanol-grown cells of these yeasts contain several large peroxisomes that harbour key enzymes of methanol metabolism, which become superfluous in fresh glucose or ethanol containing media. More recently, this process has also been described for another methylotrophic yeast, *P. pastoris* (Sakai et al., 1998; Tuttle and Dunn, 1995). In oleic-acid grown *S. cerevisiae* cells, peroxisomes that contain enzymes of the β-oxidation pathway are rapidly degraded upon shift to glucose-containing media (Chiang et al., 1996; Evers et al., 1991; Hutchins et al., 1999). In the yeast *Y. lipolytica*, selective glucose-induced peroxisome degradation was observed upon a shift of acetate/oleate/ethylamine grown cells to glucose/ammonium sulphate-containing media (Gunkel et al., 1999). In these cells, peroxisomes contain amine oxidase and catalase together with enzymes of the β-oxidation pathway and glyoxylate cycle, which become superfluous when glucose and ammonium sulphate are available as carbon and nitrogen sources.

Remarkably, selective peroxisome degradation is only induced in yeasts by shifting the carbon source. Thus, in cells grown in the presence of primary amines or D-amino acids as nitrogen sources, peroxisomes are not degraded upon a shift to ammonium sulphate as sole nitrogen source.

Finally, degradation has been observed of yeast peroxisomes that have become non-functional due to treatment of whole cells with specific chemicals that affect either peroxisomal matrix enzymes (e.g., KCN, Van der Klei et al., 1989) or the peroxisomal membrane (e.g., toxin T-514, Sepulveda-Saveedra et al., 1992; Fig. 10). The selective degradation of peroxisome in *P. pastoris* and *H. polymorpha* will be discussed in detail in the following.

Mechanisms of Peroxisome Degradation

In *H. polymorpha* both glucose and ethanol-induced peroxisome degradation involves three distinct steps: (1) tagging followed by sequestration of the organelle to be degraded by, most likely, ER-derived membranous layers, (2) heterotypic fusion of the sequestered compartment with the vacuole, and (3) degradation of the organellar contents in the vacuole (Figs. 4 and 9). Also, *H. polymorpha* peroxisomes that have been damaged by treatment of whole cells with a specific toxin (T514) are degraded by this mechanism (Sepulveda-Saveedra et al., 1992). However, when *C. boidinii* cells are incubated with T514, the whole peroxisomal population is degraded at once by uptake in the vacuole (Fig. 10). On the other hand, when *H. polymorpha* cells are placed under nitrogen starvation conditions, peroxisomes are engulfed by protrusions of the vacuolar membrane, a process that strongly resembles microautophagy in mammalian cells (Bellu et al., unpublished results).

For *P. pastoris*, different modes of peroxisome degradation have also been described. In this yeast, the mechanism of peroxisome turnover changes with the growth substrate by which peroxisome degradation is induced. Exposure of methanol-grown *P. pastoris* cells to ethanol results in degradation of individual peroxisomes by a mechanism similar to that described above for glucose or ethanol induced peroxisome degradation in *H. polymorpha*. In *P. pastoris*, glucose-induced degradation, however, results in the engulfment and subsequent uptake of clusters of peroxisomes in the central vacuole. The latter process is non-selective compared to ethanol-induced peroxisome degradation in *P. pastoris* because together with the clusters of peroxisomes, portions of the cytosol are also sequestered by the vacuolar protrusions and subsequently degraded.

The finding that *P. pastoris* peroxisomes are degraded by different pathways dependent on the inducing carbon source (ethanol vs. glucose) is corroborated by the isolation of mutants that are defective in glu-

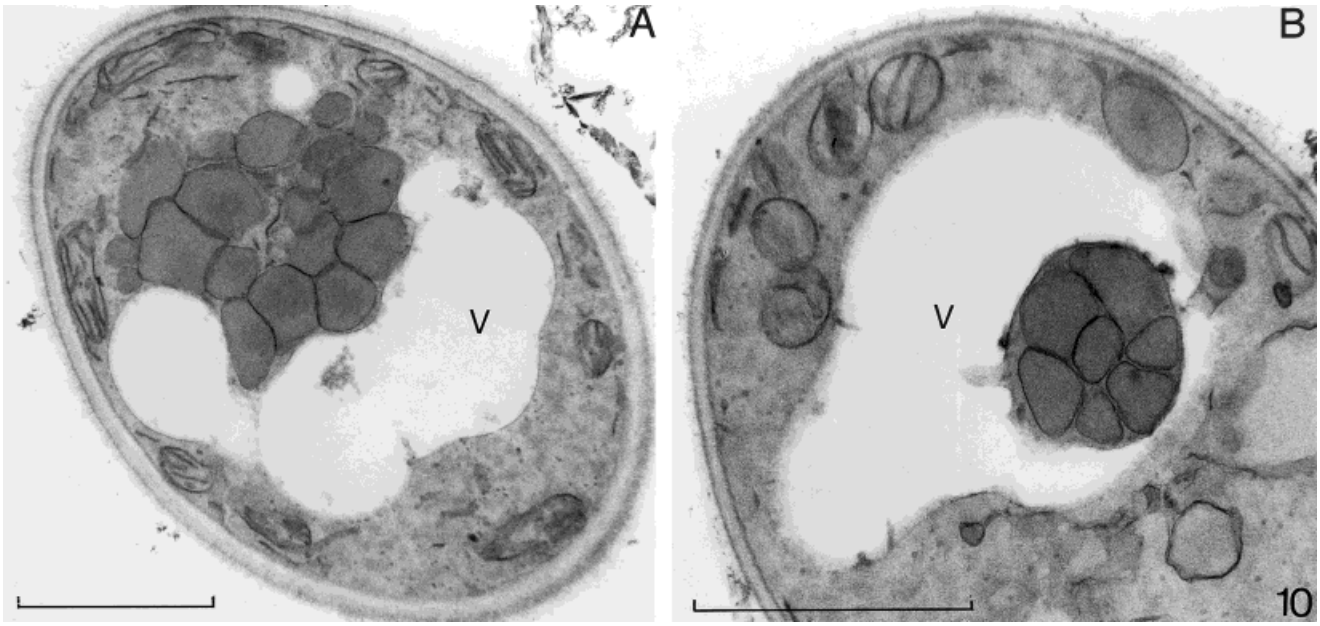

Fig. 10. Two stages of the uptake of the total population of peroxisomes in cells of *Candida boidinii*, incubated with the plant toxin T-514.

cose-induced autophagy, but are still capable to degrade peroxisomes upon exposure to ethanol. Moreover, different inhibitors (cycloheximide, PMSF, wortmannin) affect ethanol and glucose induced peroxisome degradation differently in *P. pastoris* (Sakai et al., 1998; Tuttle and Dunn, 1995).

The mechanism by which peroxisomes of *Y. lipolytica* (Gunkel et al., 1999) or *S. cerevisiae* (Hutchins et al., 1999) are taken up by vacuoles during glucose-induced degradation has not been established yet.

Mutants Defective in Peroxisome Degradation

H. polymorpha, P. pastoris, and *Y. lipolytica* mutants affected in the peroxisome degradation have been isolated in different laboratories during the last 5 years (Gunkel et al., 1999; Sakai et al., 1998; Titorenko et al., 1995; Tuttle and Dunn, 1995). *H. polymorpha* and *P. pastoris* mutants have been isolated using a colony assay based on the visualisation of the activity of the peroxisomal enzyme AO. Mutagenised cells were first grown on methanol plates to induce AO containing peroxisomes, followed by transfer of the colonies to glucose- or ethanol-containing plates. Upon incubation for a few hours, the colonies were overlayed with an AO activity assay mixture that allowed selection of those mutants that had maintained AO activity and hence were potential peroxisome degradation deficient mutants (Titorenko et al., 1995; Tuttle and Dunn, 1995). By similar techniques, peroxisome-degradation deficient mutants were isolated from *Y. lipolytica*. In this case, colonies grown on acetate/oleate/ethylamine plates were overlayed with liquid glucose/ammonium sulphate-medium and incubated for 6 hours. Subsequently, the glucose-medium was removed and replaced by an amine oxidase assay mixture that allowed visualisation of the activity of this peroxisomal enzyme (Gunkel et al., 1999).

For the isolation of *Pichia pastoris* mutants, Sakai et al. (1998) used engineered strains that produce peroxisome-bound green fluorescent protein (GFP containing a PTS1). In combination with a red fluorescent styryl dye that specifically marks the vacuolar membrane, mutants affected in glucose-induced microautophagy were selected by double-fluorescence microscopy.

Hansenula polymorpha Mutants Affected in Peroxisome Degradation

H. polymorpha mutants defective in peroxisome degradation are designated *pdd* (peroxisome degradation deficient; Titorenko et al., 1995). The *H. polymorpha pdd* mutants isolated thus far belong to 10 complementation groups (Titorenko et al., 1995; Bellu et al., unpublished results). Electron microscopical analysis revealed that all of them are blocked at initial stages of the peroxisome degradation process (sequestration of the individual organelles or fusion of enwrapped organelle with the vacuole). Moreover, the *H. polymorpha pdd* mutants are invariably defective in both glucose- and ethanol-induced degradation, suggesting that these morphologically similar processes required the same genes.

Although in *H. polymorpha* peroxisomes are degraded by a mechanically distinct process under nitrogen starvation, some of the isolated *pdd* mutants are defective in this process as well (e.g., *pdd1, pdd7*). This suggests that both processes have overlapping steps that require common genes. However, other *pdd* mutants are specifically defective in glucose and ethanol induced selective peroxisome degradation and still capable to degrade these organelles under nitrogen starvation conditions (Bellu et al., unpublished results). Hence, glucose- or ethanol-induced peroxisome degradation in *H. polymorpha* requires unique genes that do

not play a role in general, non-selective autophagy in this organism.

Mutants belonging to the *pdd1* complementation group are affected in an early stage of selective peroxisome degradation, namely the sequestration of individual organelles from the cytosol. The corresponding gene, *PDD1* (Kiel et al., 1999), is similar to *S. cerevisiae VPS34*, a gene involved in vacuolar protein sorting (Herman and Emr, 1990) and endocytosis (Munn and Riezman, 1994). The translation product of VPS34 is activated by another Vps-protein, Vps15. In addition, *VPS15* was recently shown to be essential for peroxisome degradation in *P. pastoris* (Stasyk et al., 1999).

Vps15p belongs to the serine/threonine family of protein kinases, whereas Vps34p is a phosphatidyl inositol 3-kinase. Vps15p recruits Vps34p to a yet unidentified, intracellular membrane, where Vps34p phosphorylates phosphatidyl inositol (PtdIns) molecules in the lipid bilayer. As a result, patches of PtdIns-3-P are formed, which are thought to be important for binding of other effector molecules (De Camilli et al., 1996). Because Vps34p/Vps15p plays a role in several vacuolar sorting pathways (vacuolar protein sorting, endocytosis, selective peroxisome degradation, non-selective autophagy), different effector molecules may be involved in these processes.

PDD7 encodes a gene homologous to *APG1*, a gene implicated in autophagy in *S. cerevisiae* (Matsuura et al., 1997; Komduur and Veenhuis, unpublished results). The other *H. polymorpha pdd* mutants are currently used to clone novel *H. polymorpha PDD* genes by functional complementation.

Peroxisome Degradation Defective Mutants of *Pichia pastoris*

In two laboratories, *P. pastoris* mutants defective in glucose-induced peroxisome degradation have been isolated. This process, which is characterised by the engulfment of clusters of peroxisomes by finger-like protrusions of the vacuole, can be divided into four different stages: (1) signalling, (2) sequestration by vacuolar protrusions, (3) homotypic fusion of the vacuolar membrane, and (4) degradation.

In the laboratory of Dunn, mutants have been isolated that are affected in either one of these stages. These mutants are designated *gsa* mutants, which is an acronym for glucose induced selective autophagy) (Tuttle and Dunn, 1995). Mutants affected in stage 4 turned out to be defective in vacuolar proteases (Proteinase A or B) that are involved in processing of the inactive pro-forms of newly synthesised vacuolar enzymes. Hence, defects in these proteases result in a deficiency in all vacuolar proteases.

Gsa1 and *gsa11* are blocked in the initial steps of peroxisome degradation (stage 1), *gsa9*, *10*, and *11* are blocked in stage 2, whereas *gsa7* is defective in the final homotypic fusion event (stage 3).

P. pastoris GSA1 encodes the regulatory subunit of the glycolytic enzyme phosphofructokinase 1 (*PFK1*; Yuan et al., 1997). Mutational analysis revealed that the catalytic role of this enzyme in glucose metabolism is not required for peroxisome degradation, suggesting that Pfk1p fulfills an additional function. *GSA7*, which is required for completion of sequestration of peroxisomes by the vacuole (stage 5), is a functional homologue of *S. cerevisiae APG7*. Apg7p was initially identified as a protein involved in general autophagy in *S. cerevisiae*. These findings are, therefore, another indication that selective peroxisome degradation and general autophagy share common genes. *GSA7*/*APG7* encode a unique protein with limited sequence homology to the family of ubiquitin-activating enzymes, E1 (Yuan et al., 1999).

In the laboratory of Subramani, so far 6 complementation groups of *P. pastoris* mutants (designated *pag*) were isolated that are affected in glucose-induced peroxisome degradation. Whether any of these groups overlaps with the *gsa* mutants is not yet known (Sakai et al., 1998).

CONCLUDING REMARKS

In the last decade, considerable progress was made in elucidating processes involved in peroxisome biogenesis by the identification of *PEX* genes, the subcellular localisation of the peroxins, and interactions between these peroxins. From these achievements, we obtained indications that certain peroxins and their interacting partners are involved in peroxisomal matrix protein import, membrane protein insertion, membrane transport, or organelle proliferation. More detailed studies on binding regions within peroxins and quantitative binding analyses together gave insights into the function of peroxins. However, the evidence obtained so far with the binding studies, especially on components of the matrix protein import machinery (two hybrid analysis, co-immunoprecipitations, ligand blot overlay assays), revealed that peroxins interact with each other, but it remains unclear when and where these interactions occur in the process. For example, it is thought that the matrix protein import machinery comprises a cascade of specific interactions (docking of the Pex5p/cargo complex, translocation, recycling of Pex5p), demanding one or more complexes of peroxins in consecutive steps. So far, this view remains only speculative. However, some indications have been obtained from the subcellular localisation of Pex5p in a number of *pex* mutants: *H. polymorpha pex4* (Van der Klei et al., 1998) and *pex14* (Salomons et al., 2000); *S. cerevisiae pex8* (Rehling et al., 2000); human *pex10* and *pex12* (Chang et al., 1999). In particular, biophysical techniques are now required to solve the various protein interactions in detail.

Recently, we obtained evidence for protein complexes in the peroxisomal membrane of *H. polymorpha*. Using blue native gel-electrophoresis, we could detect several protein complexes of different sizes ranging from 100 kDa to >600 kDa (Lutz et al., unpublished results). Moreover, Pex14p was detected in an approximately 400–500 kDa complex, which also contained other proteins. The identity of these proteins remains to be elucidated. With this technique, it becomes within reach to purify and identify protein complexes containing peroxins, which could be involved in a specific action in peroxisomal matrix protein import or in other processes.

Another intriguing question is whether peroxisome biogenesis and degradation of mature organelles are connected by a component, which is involved in both processes. The peroxin Pex14p could be a possible candidate. An indication for this is the finding that most

peroxisomal remnants in *H. polymorpha pex* mutants, except in the *pex14* mutant, were susceptible for glucose-induced proteolytic degradation (Veenhuis et al., 1996). Moreover, Komori et al. (1999) demonstrated that two isoforms of Pex14p exist: a phosphorylated and non-phosphorylated one. In our lab, we found that the non-phosphorylated form of Pex14p is mainly present in *H. polymorpha* wild-type cells during early exponential growth, while only the phosphorylated form of Pex14p is actively degraded after induction of peroxisome degradation (Bellu and Salomons, unpublished results). This suggests that a connection might exists between the phosphorylation-state of Pex14p and peroxisome biogenesis and degradation. However, further experiments have to be performed to elucidate the regulation mechanism(s) of peroxisome biogenesis and degradation (homeostasis).

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