Chapter 1

General Introduction
Abstract
Peroxisomes are versatile organelles that fulfill important functions in eukaryotic organisms, ranging from yeast to man. In the recent past, much progress has been made in unraveling the molecular mechanisms involved in the biogenesis of this organelle. 23 different genes (PEX genes) have been identified that are involved in peroxisome biogenesis. This chapter gives a general description of peroxisome function, structure, and biogenesis. Emphasis is given to the possibility that peroxisomes are formed de novo.

Introduction
Eukaryotic cells are subdivided into distinct cellular compartments, designated organelles. Microbodies (peroxisomes, glycosomes, glyoxysomes, and hydrogenosomes) represent the most recently discovered class of organelles. Peroxisomes are devoid of DNA, and all peroxisomal proteins studied thus far are synthesized on free cytosolic polysomes (Lazarow and Fujiki, 1985). This implies that proteins, which are destined for the peroxisomal matrix or membrane, need to be imported into the organelle post-translationally via specific import machineries. The details of the mechanism by which import occurs are currently emerging from studies in different model organisms, ranging from yeast to man, performed in various research groups.

Peroxisomes represent a highly dynamic component of the cellular machinery, and the number and size of these organelles is strongly determined by cell type and physiology. Peroxisome homeostasis not only requires careful regulation of organelle biogenesis and function, but also organelle numbers. Recently, several research groups have devoted their efforts at unraveling the mechanism of peroxisome degradation.

This general introduction summarizes our current understanding of the mechanisms of peroxisome biogenesis. It will focus on the possibility that peroxisomes are derived from non-peroxisomal cellular membranes. Recent data indicate that this possibility might exist.

Peroxisome structure and function
Microbodies represent the class of most recently discovered organelles (for a review see Purdue and Lazarow, 2001). They were first observed in 1954 by Rhodin (Rhodin, 1954) and their generalized name referred to their small size, which made
them overlooked for such a long period of time. Several years later microbodies were further classified on the basis of their metabolic function and discriminated in peroxisomes, glycosomes, glyoxysomes, and hydrogenosomes. Peroxisomes range in size from 0.1 to 1 µm and are bounded by a single membrane. They can assume different shapes depending on the type of cell that is studied. They can be spherical, tubular, or even almost cubic (in methylotrophic yeasts grown on methanol), and sometimes are interconnected (“peroxisomal reticulum”). The peroxisomal membrane is the boundary between the cytosol and the protein-rich peroxisomal matrix. Although the term microbody is still used by some scientists to describe the morphological appearance of these organelles, the name peroxisome was given to a subclass of these subcellular compartments in 1966 by de Duve and Baudhuin (De Duve and Baudhuin, 1966), after biochemical analysis of their metabolic function. They established that peroxisomes are the cellular compartment where most hydrogen peroxide metabolism takes place, hence their name. Another major and well-conserved function of peroxisomes is fatty acid β-oxidation. Microbody function is often highly specified by organism and cell type and can include such diverse processes as cholesterol and ether lipid (plasmalogen) synthesis in animals, glycolysis in trypanosomes (glycosomes), photorespiration in leaves, and glyoxylate metabolism in germinating seeds (glyoxysomes) (for review, see Purdue and Lazarow, 2001). In yeasts, peroxisomes are involved in the primary metabolism of specific carbon and nitrogen sources (Veenhuis and Harder, 1988). In methylotrophic yeast species (such as Hansenula polymorpha and Pichia pastoris) the degradation of methanol and primary amines is confined to these organelles. (For reviews of peroxisome biochemistry, see: Veenhuis and Harder, 1988; van den Bosch et al., 1992.) The enzyme activities catalyzing the above processes are located in the peroxisomal matrix (lumen). In Neurospora crassa the Woronin body was recently identified as a specialized peroxisome-derived organelle (Jedd and Chua, 2000). In this filamentous fungus, these organelles function as plugs for septal pores to avoid cytoplasmic bleeding caused by mechanical damage. The importance of peroxisomes is further demonstrated by the existence of several diseases in man associated with peroxisome malfunction, the Peroxisomal Biogenesis Disorders (PBDs), some of which are lethal (reviewed in Gould and Valle (2000)). Peroxisome biogenesis is governed by the activities of a distinct class of proteins termed peroxins (Distel et al., 1996). These peroxins are encoded by PEX genes. Their function in peroxisome biogenesis and performance is detailed below.
Chapter 1

**Peroxisome proliferation**

In yeasts, several growth substrates, including alkanes, D-amino acids, oleic acid and methanol induce peroxisome biogenesis (reviewed in Veenhuis and Harder (1988)). Growth conditions, such as type of substrate used, growth rate, and oxygen availability determine the number and size of the organelles, not the amount of peroxisomal proteins synthesized (Veenhuis and Harder, 1988). An example of this is observed in *H. polymorpha* *pim* mutants, in which a major portion of peroxisomal matrix proteins is mislocalized to the cytosol. In these mutants, small peroxisomes may be present at numbers comparable to wild type cells.

An important characteristic of peroxisomes in *H. polymorpha* is that they are capable of importing matrix proteins during a limited period in their existence. Small peroxisomes grow due to incorporation of lipids and proteins; after their maturation one or a few new organelles form by fission. The mature organelle ceases to grow, while the newly formed peroxisome increases in size. This new organelle apparently has “inherited” the ability to import new components, leaving the mature organelle as an import-incompetent “enzyme bag” (Veenhuis et al., 1989; Waterham et al., 1992). The factors involved in regulation of peroxisome proliferation and maturation are largely unknown. However, the peroxisomal membrane protein Pex11p seems to play a role in peroxisome multiplication. Overproduction of Pex11p leads to proliferation of peroxisomes, while giant peroxisomes are found in a *pex11* deletion strain (Erdmann and Blobel, 1995; Marshall et al., 1995). Marshall et al. (1996) suggested that the oligomeric state of Pex11p could be instrumental in peroxisome maturation. They found monomeric Pex11p associated with small, immature organelles, while dimeric protein was present in mature ones. The exact function of Pex11p in peroxisome maturation is clearly not resolved yet. In a more recent study, a direct role of Pex11p in transport of medium chain fatty acids into peroxisomes of *S. cerevisiae* was suggested by van Roermund et al. (2000). In their view, the proliferation of organelles is caused as an effect of increased \( \beta \)-oxidation. However, Gould and co-workers challenged this view and showed that this could not be the only reason. They observed a direct effect of Pex11p levels on peroxisome numbers, even in the absence of peroxisomal metabolic activity (Li and Gould, 2002). Further research is needed to unravel the principles of peroxisome maturation and proliferation.

After peroxisomes have become redundant for growth, e.g. after a shift of cells from peroxisome-inducing to peroxisome-repressing conditions, the organelles are rapidly degraded by a selective process called pexophagy (reviewed by Bellu and Kiel). In
H. polymorpha, this process mainly targets the mature organelles, leaving the small, import-competent peroxisomes unaffected. Recently, it was shown that a protein involved in biogenesis of peroxisomes, Pex14p, also plays a role in pexophagy (Bellu et al., 2001). Pex14p might function as a molecular switch discriminating between import competence and incompetence of peroxisomes, enabling cells to swiftly adapt to new growth conditions that require new peroxisomal metabolic activities.

**Isolation of pex mutants and cloning of PEX genes**

Yeast are excellent model organisms for studies of the mechanisms of peroxisome biogenesis by virtue of the fact that mutants that are affected in this process, the so-called pex mutants, are viable. Yeast pex mutants are unable to grow on specific media that require peroxisome function (e.g. oleate or methanol) enabling easy selection. However, normal growth can occur when non-selective substrates (e.g. glucose) are used. Complementation of the pex-specific growth defect leads to the isolation of the corresponding PEX gene. Over the past two decades 23 peroxins have been identified (see Table 1, and http://www.peroxisome.org/). For many of these, both the yeast and mammalian genes have been cloned and sequenced.

Many of the pex mutants in different organisms contain peroxisomal membrane remnants, or ghosts (Santos et al., 1988; Santos et al., 2000). This is indicative of the fact that although import of peroxisomal matrix proteins is blocked in these mutants, they are still able to assemble and maintain their membrane, including (a portion of) their proteins. An increasing amount of data is accumulating contributing to our knowledge of peroxisome biogenesis, e.g. how peroxisomal matrix proteins are targeted to their subcellular localization.

**Function and interactions of peroxins**

**Matrix protein import**

A number of the peroxins identified thus far, is involved in matrix protein import. The corresponding pex mutants contain ghosts (see above) and mislocalize peroxisomal matrix proteins to the cytosol. This clearly sets this type of mutant apart from another class, which seems to lack any detectable form of peroxisomal remnants in some organisms. These mutants (pex3, 16, 17, and 19) are affected in the biogenesis and maintenance of the peroxisomal membrane itself.

Most matrix proteins are targeted to the peroxisomal matrix by the Peroxisomal Targeting Signal (PTS) 1, consisting of a C-terminal tripeptide -SKL and its variants
Table I. Overview of peroxins

<table>
<thead>
<tr>
<th>Peroxin</th>
<th>PBD CG?</th>
<th>Protein interactions</th>
<th>Notes</th>
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<tbody>
<tr>
<td>PEX1</td>
<td>1</td>
<td>PEX6</td>
<td>AAA ATPase required for matrix protein import</td>
</tr>
<tr>
<td>PEX2</td>
<td>10</td>
<td></td>
<td>C3CH4 zinc-binding integral PMP required for matrix protein import</td>
</tr>
<tr>
<td>PEX3</td>
<td>12</td>
<td>PEX19</td>
<td>Integral PMP required for membrane biogenesis</td>
</tr>
<tr>
<td>PEX4</td>
<td>n.d.</td>
<td>PEX22</td>
<td>E2 ubiquitin-conjugating enzyme required for matrix protein import</td>
</tr>
<tr>
<td>PEX5</td>
<td>2</td>
<td>PEX7, PEX14, PEX13, PEX10, PEX12, the PTS1</td>
<td>TPR-containing PTS1 receptor, required for PTS1-mediated import (yeast) or both PTS1- and PTS2-mediated import (human)</td>
</tr>
<tr>
<td>PEX6</td>
<td>4</td>
<td>PEX1</td>
<td>AAA ATPase required for matrix protein import</td>
</tr>
<tr>
<td>PEX7</td>
<td>11</td>
<td>PEX5, PEX14, PEX13, the PTS2</td>
<td>WD-40 repeat containing PTS2 receptor required for PTS2-mediated import</td>
</tr>
<tr>
<td>PEX8</td>
<td>n.d.</td>
<td>PEX5</td>
<td>Integral PMP required for matrix protein import, contains a PTS1 as well as a PTS2</td>
</tr>
<tr>
<td>PEX9</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Integral PMP required for matrix protein import</td>
</tr>
<tr>
<td>PEX10</td>
<td>7</td>
<td>PEX12, PEX5, PEX19</td>
<td>C3CH4 zinc-binding integral PMP required for matrix protein import</td>
</tr>
<tr>
<td>PEX11</td>
<td>n.d.</td>
<td>PEX19</td>
<td>PMP involved in peroxisome proliferation</td>
</tr>
<tr>
<td>PEX12</td>
<td>3</td>
<td>PEX10, PEX5, PEX19</td>
<td>C3CH4 zinc-binding integral PMP required for matrix protein import</td>
</tr>
<tr>
<td>PEX13</td>
<td>13</td>
<td>PEX5, PEX7, PEX14</td>
<td>SH3 containing PMP required for matrix protein import</td>
</tr>
<tr>
<td>PEX14</td>
<td>14</td>
<td>PEX5, PEX7, PEX13, PEX17</td>
<td>PMP required for matrix protein import, the initial site of receptor docking</td>
</tr>
<tr>
<td>PEX15</td>
<td>n.d.</td>
<td>n.d.</td>
<td>PMP required for matrix protein import</td>
</tr>
<tr>
<td>PEX16</td>
<td>9</td>
<td>PEX19</td>
<td>Integral PMP required for membrane biogenesis</td>
</tr>
<tr>
<td>PEX17</td>
<td>n.d.</td>
<td>PEX14</td>
<td>PMP required for matrix protein import</td>
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<tr>
<td>PEX18</td>
<td>n.d.</td>
<td>PEX7</td>
<td>Required for PTS2-mediated import, thought to be involved in peroxisomal localization of PEX7, highly homologous to PEX21</td>
</tr>
<tr>
<td>PEX19</td>
<td>14</td>
<td>Multiple PMPs</td>
<td>Predominantly cytosolic, partly peroxisomal protein involved in peroxisomal membrane protein import</td>
</tr>
<tr>
<td>PEX20</td>
<td>n.d.</td>
<td>Thiolase</td>
<td>Required for thiolase import in Y. lipolytica</td>
</tr>
<tr>
<td>PEX21</td>
<td>n.d.</td>
<td>PEX7</td>
<td>Required for PTS2-mediated import, thought to be involved in peroxisomal localization of PEX7, highly homologous to PEX21</td>
</tr>
<tr>
<td>PEX22</td>
<td>n.d.</td>
<td>PEX4</td>
<td>PMP required for matrix protein import, thought to be a docking factor for PEX4</td>
</tr>
<tr>
<td>PEX23</td>
<td>n.d.</td>
<td>n.d.</td>
<td>PMP required for matrix protein import</td>
</tr>
<tr>
<td>Djp1</td>
<td>n.d.</td>
<td>n.d.</td>
<td>DnaJ-like protein involved in peroxisomal matrix protein import</td>
</tr>
</tbody>
</table>

**PBD CG:** Peroxisome Biogenesis Disorder Complementation Group

For references, see: http://www.peroxisome.org
A minor subset of matrix proteins contains a PTS2, a nonapeptide with consensus sequence (R/K)-(L/V/I)-X5-(H/Q)-(L/A) present near the N-termini of these proteins. Both PTS1 and PTS2 proteins are recognized by specific cytosolic receptors, Pex5p and Pex7p, respectively.

**Fig. 1.** Schematic representation of PTS1 matrix protein import in H. polymorpha. Several steps can be distinguished in this process, as indicated. (1) In the cytosol the PTS1 receptor Pex5p binds to the PTS1 of newly synthesized matrix proteins. (2) The Pex5p-cargo complex docks to the peroxisomal docking site, which contains Pex13p, Pex14p, and Pex17p. (3) Subsequently, Pex5p interacts with Pex12p, which in turn is in close proximity of Pex10p. Pex10p and Pex12p form the putative entry site (or translocon) for matrix protein import. (4) The Pex5p-cargo complex enters the peroxisome, after which (5) Pex5p releases from its cargo. Pex8p might aid this process, and is subsequently released in the peroxisomal matrix. (6) Finally, (7) Pex5p is recycled to the cytosol. This process involves Pex4p, which is anchored to the membrane by Pex22p. (Adapted from Veenhuis et al, 2000)

Pex5p (McCollum et al., 1993) and Pex7p (Marzioch et al., 1994) have been identified in several organisms. In a pex5 mutant the import of PTS1-containing peroxisomal matrix proteins is disturbed, while in a pex7 mutant PTS2 proteins are mislocalized to the cytosol. In man, Pex5p and Pex7p have been shown to interact
directly. This interaction is regulated at the level of alternate splicing. Human Pex5p exists in two forms, a long (Pex5pL) and a short (Pex5pS) form, differing by the presence of a 37-amino acid internal region encoded by exon 8 of the \textit{PEX5} gene. This particular region is responsible for the interaction of Pex5p with Pex7p (Braverman et al., 1998). In \textit{Yarrowia lipolytica} and \textit{Caenorhabditis elegans}, no evidence for the presence of the \textit{PEX7} gene has been found thus far. Instead, Pex20p was identified as a cytosolic factor involved in thiolase import (Titorenko et al., 1998). Pex20p plays a role in dimerization and transport of thiolase, and was shown to interact with Pex8p (Smith and Rachubinski, 2001).

The interaction of Pex5p with PTS1-containing proteins has been studied in detail. Several groups have shown independently, that the tetratricopeptide repeats (TPR) present in the C-terminal two-thirds of Pex5p, bind the PTS1 (Brocard et al., 1994; Fransen et al., 1995; Terlecky et al., 1995; Szilard and Rachubinski, 2000). Detailed information on binding of PTS1 by the TPR domains came from the 3-dimensional structure analysis of the C-terminus of human Pex5p (Gatto, Jr. et al., 2000), and from a mutational analysis of \textit{S. cerevisiae} Pex5p (Klein et al., 2001) The localization of both receptors has been a matter of debate for some time. In different studies performed in several species, both proteins have been found in the cytosol, in the peroxisomal matrix, or in both (Dodt et al., 1995; van der Klei et al., 1995; Szilard et al., 1995; Zhang and Lazarow, 1995; Elgersma et al., 1998). A dual localization of the receptors could indicate that import of receptor-cargo complexes takes place via the so-called ‘shuttling-receptor’ model. In this model, the receptor protein, after binding its cargo molecule, docks to a site on the peroxisomal surface and is then translocated across the membrane into the organellar matrix. After translocation, the cargo is released from the receptor and the latter is transported back into the cytosol. Recently, experimental evidence was obtained in the group of Subramani, which indicates that this model is true for human organelles (Dammai and Subramani, 2001). Whether Pex7p behaves in a similar fashion remains a matter of debate.

A third type of PTS (sometimes referred to as PTS3) was found in acyl-CoA oxidase of \textit{S. cerevisiae} and \textit{Candida tropicalis} (Small et al., 1988). \textit{S. cerevisiae} catalase A seems to contain both a PTS1, as well as an internal PTS3. Using the yeast two-hybrid system as well as \textit{in vitro} binding studies, Skoneczny and Lazarow found an interaction between \textit{S. cerevisiae} acyl-CoA oxidase and Pex5p, the PTS1 receptor, suggesting a role for the PTS1 receptor in PTS3 import (Skoneczny and Lazarow, 1998).
The factors involved in recycling of Pex5p remain unknown thus far. However, in *H. polymorpha*, Pex4p seems to play a direct role in recycling of Pex5p. Overexpression of *PEX5* in a *pex4* strain leads to the accumulation of Pex5p in the peroxisomal matrix (van der Klei et al., 1998). Pex4p is a member of the E2 family of ubiquitin-conjugating enzymes (Wiebel and Kunau, 1992). This protein is anchored to the peroxisomal membrane by Pex22p, an integral membrane protein (Koller et al., 1999). Both Pex4p and Pex22p are essential for PTS1 and PTS2 import; in *H. polymorpha* however, Pex4p is dispensable for PTS2 import (van der Klei et al., 1998). The target molecule for ubiquitination by Pex4p is unknown, although it was hypothesized that Pex18p and Pex21p might be the targets in *S. cerevisiae*. A double knock-out ∆pex18/∆pex21 strain of baker’s yeast shows an import defect of PTS2 proteins, whereas PTS1 import occurs normally (Purdue et al., 1998). A single deletion of either of the genes only has a small impact on PTS2 import. Both proteins are very unstable *in vivo* (half-life of <10 min; (Purdue and Lazarow, 2001)), and Pex18p becomes mono- and di-ubiquinated during peroxisome biogenesis. Possibly, this ubiquitination of Pex18p represents the link between Pex4p activity and receptor recycling (see above). However, it should be noted that *H. polymorpha* Pex4p is exclusively involved in PTS1 import (van der Klei et al., 1998).

After binding of proteins destined for the peroxisomal matrix to their respective receptors, the next step in the sequence of events leading to import is binding of the receptor-cargo complex to a docking site localized on the cytosolic face of the peroxisomal membrane. This role is fulfilled by Pex13p (Gould et al., 1996), Pex14p (Gould et al., 1996; Komori et al., 1997; Will et al., 1999), and Pex17p (Huhse et al., 1998). Pex13p and Pex14p were identified as binding partners for both Pex5p and Pex7p in multiple studies (Elgersma et al., 1996); (Girzalsky et al., 1999); (Fransen et al., 1998); (Shimizu et al., 1999). Since both Pex5p and Pex7p bind to Pex13p and Pex14p, the docking complex is actually the site were the PTS1 and PTS2 pathways converge. This also explains why mutations in peroxins acting downstream of Pex13p/Pex14p, typically affect both import routes. Although both proteins appear to play a role in import of matrix proteins, the *pex14* phenotype can be rescued by overexpression of Pex5p in *Hansenula polymorpha*, indicating that Pex14p’s function can be circumvented in this organism (Salomons et al., 2000). Possibly, Pex14p is only required for enhancing the efficiency of import, conceivably by directing receptor-cargo complex towards the docking site (Pex13p).

After binding of the receptor-cargo complex to the putative docking site on the cytosolic face of the peroxisomal membrane, translocation of PTS-containing matrix
proteins across the organellar membrane must take place. Little is known about the mechanism of translocation. It is tempting to speculate that the three ring-finger containing proteins, Pex2p (Tsukamoto et al., 1991), Pex10p, and Pex12p (Chang et al., 1999; Okumoto et al., 2000) (all integral components of the peroxisomal membrane) are involved in this step. The significance of the ring-finger domains for their function is not clear, however, mutational analysis of this domain indicates that it is essential for proper functioning of Pex2p in matrix protein import, but not for Pex5p docking. It has been suggested that Pex2, 10 and 12p might form the core translocon that accommodates the transfer of receptor-cargo complexes across the membrane. Such a putative peroxisomal translocon needs to meet at least two important prerequisites: First, translocation of proteins must be mediated without disrupting the solute gradients that exist across the membrane. Secondly, the translocon must be able to accommodate very large structures. It is a well-established fact that peroxisomes maintain a pH gradient (Nicolay et al., 1987; Dansen et al., 2000), suggesting that even molecules as small as protons are not able to passively traverse the translocation apparatus. This suggests that a mechanism for preventing leakage of small molecules must exist. The first possible mechanism requires that the translocation pore is effectively sealed during its activities, either by means of an external “plug” (comparable to the function of the lumenal ER-chaperone Kar2p (BiP) in transport of secretory proteins into the ER lumen (Haigh and Johnson, 2002)), or by a tightly regulated concerted closing and opening of the putative translocon itself. In another conceivable mechanism, import of matrix proteins does not occur via a translocon, but rather by a vesicle fusion event. The second prerequisite for the peroxisomal translocon (the ability to accommodate large structures) is most dramatically illustrated by the work of Walton and co-workers, who showed that gold particles, ranging in size from 4-9 nm, coated with PTS1-containing proteins and microinjected into cultured mammalian cells, could be taken up by peroxisomes (Walton et al., 1995). Other examples of import of quite large molecules into peroxisomes exist, e.g. the import of tetrameric catalase (~ 240 kDa (Brul et al., 1988)).

Two proteins belonging to the family of AAA-ATPases, Pex1p and Pex6p, are involved in peroxisome biogenesis (Erdmann et al., 1991; Faber et al., 1998). One of the many cellular functions in which AAA-ATPases play a role is membrane fusion (mammalian NSF and \textit{S. cerevisiae} Sec18p (Malhotra et al., 1988; Eakle et al., 1988)). Interestingly, Pex1p and Pex6p have been localized both to vesicles as well
as to peroxisomes. This suggests that a fusion event might occur during some stage of peroxisome biogenesis, possibly involving vesicles.

Even though we possess some information on the characteristics of the peroxisomal translocon, its nature still eludes us. When studied by electron microscopy, and more in particular freeze etch techniques, there is no sign of the presence of large protein complexes in the peroxisomal membrane (as one would expect if a large pore analogous to the nuclear pore complex would be present). Also biochemically, no clear evidence for the existence of such a complex has been found yet, although recent work in our group using blue native gel electrophoresis has provided preliminary evidence for the existence of a peroxisomal membrane protein complex.

**Membrane protein insertion**

The membrane surrounding peroxisomes contains several proteins, which are either involved in peroxisome biogenesis and maintenance (membrane-bound peroxins), or in transport and enzymatic functions (e.g. PMP70 in humans, or Pat1p, Pat2p, and PMP47 in yeasts).

Compared to our understanding of the targeting of matrix proteins to the peroxisome, little is known about insertion of membrane proteins into the peroxisomal membrane. Determining the minimal targeting information for peroxisomal membrane proteins (the mPTS) has proven to be cumbersome. In general, approaches using mutagenesis of membrane proteins harbour the intrinsic difficulty that great care must be taken to avoid misinterpretations caused by, for instance, instability of mutant proteins. Furthermore, the tendency of membrane proteins to aggregate or randomly insert into membranes after removal of their proper targeting information is cause for concern in these types of studies.

Recently, Gould and co-workers have directed their attention towards a re-evaluation of the mPTS in peroxisomal membrane proteins (PMPs). They identified two regions in human PMP34 that seem to function as mPTSs (Jones et al., 2001). They speculate that the multiple mPTSs in a single PMP might function as binding sites for a specific PMP binding factor that is required for targeting of PMPs to the peroxisomal membrane. PMPs containing more than one membrane-spanning domain (like PMP34) could harbor more than one mPTS.

*In vitro* studies on insertion of peroxisomal membrane proteins into isolated organelles have resolved some of the minimal requirements of this process. These studies have revealed two distinct steps in membrane insertion: (1) Binding of the protein to the membrane, and (2) correct insertion into the membrane (Diestelkotter
and Just, 1993; Just and Diestelkotter, 1996; Imanaka et al., 1996). Insertion of the model proteins PMP22 and PMP70 did not require ATP or GTP, whereas Pex2p-insertion required the presence of ATP, but not its hydrolysis. Protease pre-treatment of isolated organelles showed the involvement of proteinaceous factors, which are insensitive to N-ethylmaleimide. Cytosolic factors are involved in this process (Imanaka et al., 1996; Pause et al., 1997). These cytosolic factors might include molecular chaperones involved in maintaining the hydrophobic membrane proteins in an import-competent state, rather than allowing their aggregation. Pex19p seems to be the cytosolic receptor for peroxisomal membrane proteins. Pex19p was shown to bind to many peroxisomal membrane proteins. Furthermore, targeting of Pex19p to the nucleus by means of a Nuclear Localization Signal (NLS) led to accumulation of PMPs in the nuclear envelope (Fransen et al., 2001; Snyder et al., 2000).

The question of the origin of the peroxisomal membrane is analogous to the general issue of peroxisomal origin. Peroxins (or other types of proteins) directly involved in biosynthesis of the membrane of peroxisomes probably exist, but so far only circumstantial evidence for this hypothesis has been found. In particular Pex3p, is thought to be directly involved in biogenesis of the peroxisomal membrane. This notion is substantiated by the complete lack of peroxisomal remnants in a pex3 mutant, and by the observation that the integrity of the peroxisomal membrane is affected in a conditional pex3 mutant, after synthesis of Pex3p is blocked (Baerends et al., 1996).

Models for peroxisome biogenesis: Past and present

The possibility that the ER or nuclear membrane play an active role in peroxisome biogenesis, as already indicated by the early morphological work on microbodies, has been the subject of many recent studies. By now, an impressive amount of evidence has been gathered, pleading the case for an active role of the ER in peroxisome biogenesis.

Titorenko et al. discovered the existence of five peroxisomal subpopulations (P1-P5) that consist of pre-peroxisomal vesicles which develop into mature peroxisomes (P6) by a series of fusion events. This process was also reconstituted in vitro and its minimal requirements were determined. Strikingly, the same group discovered overlapping phenotypes between mutants affected in the general secretion pathway (sec23 and srp54), and pex mutants (pex1, 2, 5, 6, 8, 9, and 16). Furthermore, Pex2p and Pex16p of Y. lipolytica are N-linked core glycosylated, indicating that these proteins have been in direct contact with the ER lumen.
Overexpression of PEX genes often leads to strong proliferation of ER membranes, although Stroobants et al. (Stroobants et al., 1999) propose that this phenomenon is caused by a general stress response.

The observation that ARF and all eight subunits constituting the COPI coatomer complex bind to peroxisomes in rat liver implies the possibility of vesicles budding from the peroxisome. It might be envisaged that this process is important in fission of peroxisomes, especially since Pex11p is able to bind coatomer and ARF (Passreiter et al., 1998). Coatamer and ARF are cellular components involved in vesicle-mediated transport processes. Pex11p contains a dilysine motif, which functions in ER retention of integral membrane proteins. Dilysine motifs facilitate retrieval of ER-resident proteins from ER-Golgi intermediate compartments back to the ER.

In H. polymorpha it was shown that BFA, a fungal toxin which blocks vesicle-mediated transport, also affects peroxisome biogenesis and leads to accumulation of both peroxisomal membrane (Pex3p, Pex14p) and matrix (AOX, Pex8p) proteins to the ER (Salomons et al., 1997). Furthermore, targeting studies on Pex3p revealed that the N-terminal 16 amino acids of this protein efficiently target a marker protein to the ER (Baerends et al., 1996). When the Pex3p fragment was extended to 37 residues, the same marker protein was targeted to peroxisomes. The first 50 amino acids of Pex3p drive the formation of ER-derived vesicles in a pex3 mutant, which can function as precursors for formation of mature peroxisomes after re-introduction of full-length Pex3p (Faber et al., 2002; see chapter 4). Whether this phenomenon indicates a pathway that is significant in WT H. polymorpha in vivo, or whether it represents a special rescue mechanism that only occurs in the absence of peroxisomal membrane structures remains to be resolved. Recently, Hazra et al. identified the presence of peroxisomal remnants in a P. pastoris pex3 knockout strain (Hazra et al., 2002). This indicates that in this organism rescue of peroxisome biogenesis in a pex3 background need not occur via an alternative route, but rather proceeds by incorporating proteins and lipids into the remnants.

Taken together, there is now much evidence indicating the existence of a pathway other than the “growth and fission” model for biogenesis of peroxisomes. However, the issue is still matter of vigorous debate. New and more conclusive evidence will be needed to decide whether the proposed alternative pathway really plays a role in peroxisome biogenesis in vivo.
Chapter 1

Aim and outline of this thesis

The objective of this study was to analyse the early events of peroxisome biogenesis in the methylotrophic yeast Hansenula polymorpha. This approach was inspired by the observation that specific mutants of this organism completely lack detectable peroxisomal remnants, but nevertheless are able to form new organelles shortly after the specific defect was complemented. The analysis of initial development of these newly formed organelles was expected to further deepen our insight into the cellular origin of peroxisomes.

In order to expand the existing possibilities to introduce gene constructs into H. polymorpha, either for industrial or scientific purposes, we set out to develop a new expression system exploiting a H. polymorpha auxotrophic mutant. Chapter 2 describes the cloning of two genes, PUR7 and PUR8, and the further exploitation of the PUR7 gene to construct pHIPA4. It is shown that pHIPA4 is well suited for mild overexpression of genes.

The pex3 mutant of H. polymorpha completely lacks detectable peroxisomal remnants. When the function of the PEX3 gene is restored, new organelles appear within a short time span. Therefore, we assume that the Pex3 protein might play an important role in the early events of peroxisome biogenesis. We first studied the localization of Pex3p in detail, as described in chapter 3. The results show that H. polymorpha Pex3p is not an integral protein as suggested previously. Instead, it is tightly bound to the cytosolic face of the peroxisomal membrane. Furthermore, using GFP as fluorescent marker, distinct focal concentrations of Pex3p.GFP were observed at the peroxisomal membrane, suggesting that Pex3p is present in protein clusters.

In chapter 4 the events occurring after reintroduction of a 50 amino acid long N-terminal fragment of Pex3p fused to GFP into the pex3 mutant are studied. Surprisingly, large numbers of vesicles accumulate in this situation that contain N50 Pex3p. We show that the vesicles originate from the nuclear membrane and possess a number of peroxisomal characteristics. Also, these vesicles are the sole target of subsequently reintroduced full-length Pex3p and serve as templates for the biogenesis of peroxisomes.

In continuation of this, the research described in chapter 5 provides evidence for the possibility that peroxisomes are formed at the nuclear membrane in pex3 cells after reintroduction of the complete PEX3 gene.
References


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