Review
Import of proteins into peroxisomes
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Abstract

Peroxisomes are organelles that confine an important set of enzymes within their single membrane boundaries. In man, a wide variety of genetic disorders is caused by loss of peroxisome function. In the most severe cases, the clinical phenotype indicates that abnormalities begin to appear during embryological development. In less severe cases, the quality of life of adults is affected. Research on yeast model systems has contributed to a better understanding of peroxisome formation and maintenance. This framework of knowledge has made it possible to understand the molecular basis of most of the peroxisome biogenesis disorders. Interestingly, most peroxisome biogenesis disorders are caused by a failure to target peroxisomal proteins to the organelar matrix or membrane, which classifies them as protein targeting diseases. Here we review recent fundamental research on peroxisomal protein targeting and discuss a few burning questions in the field concerning the origin of peroxisomes. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Peroxisome; Protein; Membrane

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1. Introduction

Peroxisomes are cellular organelles bounded by a single membrane that characteristically house the enzymes for fatty acid β-oxidation, hydrogen-peroxide-producing oxidases and catalase. Their importance in human metabolism is illustrated by the occurrence of severe genetic disorders caused by disturbances in peroxisome biogenesis and metabolism. Peroxisomes belong to the morphologically designated group of eukaryotic organelles known as microbodies which also includes glyoxysomes in plants [1] and fungi [2] and glycosomes in Kinetoplastida [3]. Both the mechanism by which proteins are targeted and imported into microbodies and the components required for peroxisome biogenesis (peroxins; abbreviated as pex) are evolutionarily conserved [4].

The biogenesis of peroxisomal matrix proteins is understood increasingly well (for recent review see [5–8]). Peroxisomal proteins are nuclear-encoded and are synthesised in the cytoplasm on free polyribosomes [9]. Most peroxisomal matrix proteins are synthesised at their mature molecular weight and contain either a carboxy-terminal or an amino-terminal peroxisomal targeting signal (PTS1 or PTS2, respectively). The peroxisomal targeting signals (PTS) are recognised by specific receptors (for review see [5,8,10]). These proteins are thought to act as receptors that cycle between the cytoplasm, where they bind PTS-containing matrix proteins, and the peroxisomal membrane, where they deliver their cargo. After binding of the targeting complex to peroxisomal membrane, both PTS1- and PTS2-containing proteins are translocated across the peroxisomal membrane via a shared translocation apparatus, upon which they reach the peroxisomal matrix. Peroxisomal membrane proteins are targeted and inserted post-translationally into the peroxisomal membrane via a different pathway than peroxisomal matrix proteins. However, some peroxisomal membrane proteins have been suggested to reach their location via the endoplasmic reticulum (for review see [11,12]).

We will review recent progress concerning the import of peroxisomal proteins and the targeting and insertion of membrane proteins. Furthermore, we will discuss recent views with respect to the origins of peroxisomes. We will refer to comprehensive reviews for older work that has been incorporated, but not explicitly cited.

2. Experimental approaches

In order to study the peroxisomal protein import process, two complementary approaches are being followed. (1) Genetic screens in yeast, mammals and Kinetoplastida have identified 22 factors (peroxins, see Table 1) that are mutated in peroxisome assembly mutants [8,13–16]. (2) In vitro import assays are being developed in order to identify factors involved in the import process and to test the function of the various peroxins. These in vitro import assays have been developed successfully for plant peroxisomes [17–21]. Unfortunately, hardly any plant peroxins have been identified that can be tested in these systems. Recently, however, Baker and co-workers developed an in vitro import system with sunflower glyoxysomes and purified recombinant protein. They could distinguish intermediates on the import pathway, which opens the way to detailed analysis of the import process and identification of proteins which may associate with the import intermediate [21].

In yeast and mammalian cells, in vitro import systems have been difficult to reproduce. The major
problems encountered include fragility of peroxisomes and the lack of a solid criterion for import. In contrast to the import of precursor proteins into the endoplasmic reticulum, chloroplasts and mitochondria, import of peroxisomal proteins is not coupled to processing (see also below) and therefore import has been addressed only by protease-protection (for review see [22]). This stimulated the development of permeabilised cell systems or microinjection-based import assays [23–26]. These studies plus the in vitro import studies revealed that the import process can be divided into two steps: (a) an ATP-independent binding step to the peroxisomal membrane which can occur at low temperature (0°C) and which is signal-dependent and (b) a translocation step which is energised; by ATP hydrolysis and which is temperature-dependent. Furthermore, these studies showed that matrix protein import is a saturable process which depends on the presence of membrane-associated factors and which can occur independent of a membrane potential. Although in most studies a requirement for cytosolic factors was found, in some studies addition of cytosol had no stimulatory effect (for review see [17,22]). The lack of a cytosolic requirement could be caused by the presence of cytosolic factors in the translation mixture or by copurification of cytosolic factors with peroxisomes, or by a bypass of cytosolic factors when using a high concentration of import substrate [21].

3. Peroxisomal matrix protein import

3.1. Peroxisomal targeting signals

Peroxisomal matrix proteins are nuclear encoded, are synthesised on free polyribosomes and are released into the cytoplasm before they are imported [9]. Specific targeting of most peroxisomal matrix proteins is achieved by an evolutionarily conserved C-terminal tripeptide of the sequence serine-lysine-leucine or a conserved derivative thereof (consensus sequence; S/C/A,K/R/H,L). This amino acid sequence, called the peroxisomal targeting signal type I (PTS1) has been found to be both necessary and sufficient to direct reporter proteins to peroxisomes [27,28]. However, various peroxisomal matrix proteins contain a C-terminal sequence with a two-out-of-three fit with the consensus sequence and some of these proteins are imported in one species but not in another. For instance, the PTS1 of human alanine: glyoxylate aminotransferase comprises -KKL, a signal that is not able to target a reporter protein to human peroxisomes [29]. The PTS1 could be changed into several other non-consensus PTS1 sequences without interference of targeting capacity [29]. Extensive variation of the three C-terminal amino acids of the Saccharomyces cerevisiae protein Mdh3p revealed that a much greater variety of PTS1 variants allowed import of Mdh3p into yeast peroxisomes than the consensus PTS1 sequence would predict [30]. These results imply that besides the C-terminal tripeptide additional information within peroxisomal proteins is required for import. It has now become clear that the amino acids directly preceding the PTS1 provide the additional information [31–33]. Lametschwandtner et al. [33] showed, by making use of the two-hybrid system, that the PTS1 receptor is able to bind a wide variety of C-terminal tripeptides and that the strength of the interaction is modulated by the preceding eight amino acid residues. Therefore, the PTS1 is not confined to the three C-terminal amino acids of peroxisomal matrix proteins but includes also the amino acids just upstream. Most likely, this region binds in a single binding site on the PTS1 receptor and multiple contacts are required for efficient binding. In the case of the consensus tripeptide sequence, the three C-terminal amino acids bind the receptor with such a high affinity that no additional interactions with the PTS1 receptor are required. On the other hand, non-consensus tripeptides depend on the interaction of upstream amino acid residues with the PTS1 receptor (see Fig. 1). Indeed, all non-consensus PTS1-containing human proteins contain a lysine residue immediately preceding the C-terminal tripeptide (see Fig. 1). The human PTS1 receptor and S. cerevisiae PTS1 receptor recognise a partially overlapping set of tripeptides, and the positions of the upstream amino acids that contribute to PTS1 recognition vary between man and yeast. Therefore, the observation that some peroxisomal proteins are imported in a species-specific manner can be attributed to differences in the PTS1 receptors to recognise the C-terminal tripeptide and its accessory sequences [33].

The PTS2 is found in only a few peroxisomal ma-
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<tr>
<th>Peroxin</th>
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<tr>
<td>Pex1p</td>
<td>AAA ATPase</td>
<td>Interacts with Pex6p</td>
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<td>Pex2p</td>
<td>C3HC4 motif (=RING domain), PMP</td>
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<td>Pex3p</td>
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<td>Pex4p</td>
<td>Ubiquitin conjugating enzyme, peroxisome-associated</td>
<td>PTS1 receptor accumulates in peroxisomes of <em>H. polymorpha v</em>&lt;sup&gt;pex4&lt;/sup&gt; cells</td>
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<td>Pex5p</td>
<td>PTS1 receptor, 7–8 TPR motifs, cytosol and peroxisome</td>
<td>PTS1-specific cycling receptor, binds PTS1 and the docking complex</td>
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<tr>
<td>Pex6p</td>
<td>AAA ATPase</td>
<td>Required for stability of PTS1 receptor in human fibroblasts, interacts with Pex1p</td>
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<td>Pex7p</td>
<td>PTS2 receptor, 6 WD-40 motifs, cytosol and peroxisome</td>
<td>PTS2-specific import factor that binds PTS2, Pex18p, Pex21p and the docking complex</td>
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<td>Pex8p</td>
<td>Peroxisomal membrane-associated, contains PTS1</td>
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<td>Pex10p</td>
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<td>Pex17p</td>
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<td>Pex19p</td>
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<td>Pex20p</td>
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<td>Djp1p</td>
<td>J domain, cytosol</td>
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For unified nomenclature see [13]. For references and a more detailed description of most the peroxins see text or [8].
trix proteins in yeast, plants, mammals and Kineto-plastida (for review see [34,35]) The PTS2 is a bipartite signal of the following consensus: (R/K)(L/V/I)X5(H/Q)(L/A). Although this sequence is found mainly at the N-terminus of proteins, it can function internally [36]. In mammals and plants, PTS2 proteins are synthesised as precursors, with the PTS2 residing in the cleavable presequence [37–43]. In contrast to biogenesis of mitochondrial and secretory precursor proteins, import of the precursor and cleavage of the presequence are not coupled [44].

Various examples of internal targeting signals have been reported, but they remain ill defined [45–48]. Are these internal peroxisomal targeting signals reflecting the existence of a novel import pathway with its own unique import component(s) or are they recognised by the PTS1 or PTS2 receptors? Interestingly, S. cerevisiae catalase A and carnitine acetyltransferase are both mislocated to the cytosol in cells lacking the PTS1 receptor. Furthermore, carnitine acetyltransferase lacking its PTS1 could still interact with the PTS1 receptor in a yeast two-hybrid assay. These results show that import mediated via the internal PTS of these peroxisomal matrix proteins depends on the PTS1 receptor [48]. For S. cerevisiae acyl-CoA oxidase, however, it remains un-

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**Fig. 1.** Model for PTS1 recognition by the PTS1 receptor. (A) Consensus PTS1 tripeptides (S,A,C/K,R,H/L) interact strongly in the PTS1-binding site, without the need for additional interactions between the receptor and the PTS1-containing protein. Non-consensus PTS1 tripeptides function as targeting signals only in combination with additional interactions with the PTS1 receptor. (B) In all human peroxisomal proteins that contain a non-consensus PTS1, the C-terminal tripeptide is preceded by a lysine residue (K). KANL of human catalase is sufficient to direct a reporter protein to peroxisomes, whereas ANL is not [31].
clear how it reaches the peroxisomes. The group of Lazarow isolated *S. cerevisiae* mutants that were disturbed in either PTS1 import or PTS2 import. They reported that import of acyl-CoA oxidase is neither disturbed in PTS1-import-deficient cells nor in PTS2-import-deficient cells. This observation lead them postulate the existence of a third peroxisomal protein import pathway [49].

Alternatively, subunits from homodimeric or homomeric peroxisomal matrix proteins lacking a PTS can be imported into peroxisomes in complex with a PTS-containing subunit [30,50–52]. It has therefore been postulated that some proteins (for instance acyl-CoA oxidase) might enter peroxisomes by association with PTS-containing proteins forming a heteromeric complex (for review see [5,6,35]). If this occurs, a multimerisation sequence would be interpreted as a PTS.

### 3.2. Protein folding and assembly

It is now generally accepted that at least some proteins fold and oligomerise in the cytoplasm prior to import into peroxisomes and that complete unfolding is not a prerequisite for translocation (for review see [6]). This is based on the following observations: (1) peroxisomal matrix proteins can fold and assemble into their active conformation in the cytosol in peroxisome assembly mutants; (2) subunits lacking a PTS can be piggy-back-imported with PTS-containing subunits after oligomerisation in the cytoplasm [30,50–53]; (3) the subunits do not seem to mix during the transport process, suggesting that oligomer dissociation is not required for translocation; (4) import of a DHFR fusion protein is not inhibited in vivo by the conformation-stabilising ligand aminopterin [54]; (5) chemically cross-linked human serum albumin (HSA) conjugated to multiple PTS1-containing peptides was imported into fibroblast peroxisomes after microinjection into the cytoplasm [55]; and (6) even 9-nm gold particles coated with HSA-PTS1 peptides were incorporated into peroxisomes [55].

Although these studies imply that folding and assembly might occur prior to import and that the import machinery can handle folded proteins as substrate, it does not necessarily imply that this is the way peroxisomal matrix proteins are normally imported. Indeed, it is now clear that proteins do not need to be in their native conformation to be im-

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**Fig. 2. Model showing the two pathways for peroxisomal protein targeting.** The PTS-specific soluble receptors Pex5p and Pex7p recognise PTS-containing proteins in the cytoplasm. The loaded receptors subsequently bind the peroxisomal membrane docking complex that consists of Pex13p, Pex14p and Pex17p. The docking complex is required for both PTS1 and PTS2 protein import.
ported. Human alanine:glyoxylate aminotransferase I (AGT) can be imported both as a homodimer and as a monomer [53]. Furthermore, in vitro import studies into pumpkin glyoxysomes revealed that monomeric isocitrate lyase was imported more efficiently than the homotetramer [20]. Alcohol oxidases of the yeast Hansenula polymorpha, Candida boidinii and Pichia pastoris are targeted to peroxisomes as unassembled monomers [56–58]. Assembly of alcohol oxidase into homo-octamers has been proposed to occur in the peroxisomal matrix [56,59,60].

3.3. Molecular chaperones

Molecular chaperones act by binding to non-native substrate proteins in stoichiometric amounts, keeping them in a soluble state and preventing non-productive interactions (for review see [61]). Molecular chaperones of the 70-kDa heat shock protein family are involved in a variety of different processes including protein folding, disassembly of oligomeric protein complexes, delivery of proteins to proteases and translocation of polypeptides across intracellular membranes. The diverse actions of Hsp70s are regulated by Hsp40s, J-domain-containing proteins that recruit Hsp70 to their site of action. The J-domain binds a specific Hsp70 family member, whereas other parts of the J-domain-containing protein are thought to interact with the HSP70 substrate (for review see [62,63]).

A requirement for a Hsp70/Hsp40 chaperone system in peroxisomal protein import is now well established. Import of a folded peroxisomal matrix protein was inhibited by an antiserum against the cytoplasmic Hsp73 in a permeabilised-cell assay and in a microinjection-based import assay into human fibroblasts [64]. They reported also that Hsp73 was found associated with the peroxisomal surface in rat hepatocytes, especially under conditions of peroxisome proliferation.

Import of isocitrate lyase requires the presence of Hsp70 in an in vitro glyoxysomal import assay [20]. Furthermore, a prenylated Hsp40 and an Hsp70 have been shown associated with plant glyoxysome membranes [20,65,66]. Recently, we have reported the identification of a cytosolic J-domain-containing protein (Djp1p) that is specifically required for peroxisomal protein import in S. cerevisiae [67]. Cells lacking the DJP1 gene partially mislocalise peroxisomal matrix proteins to the cytoplasm. The cytosolic location of Djp1p and its specific role in peroxisomal protein import are intriguing and suggest an early role in the targeting process. Whereas the dependence of Hsp70 for import of glyoxysomal isocitrate lyase into glyoxysomes was during protein translation [20], Hsp73 and Djp1p are required at a post-translational step [64,67].

What is the post-translational role of the Hsp40/Hsp70 machinery in peroxisomal protein import? Although Hsp40s and Hsp70s have been implicated in the folding and assembly of proteins, we consider it unlikely that Djp1p is required for folding of peroxisomal matrix proteins into their native conformation in the cytoplasm since mislocalised enzymes are active in Δdjp1 cells. Furthermore, as mentioned above, acquiring a native conformation does not necessarily imply that the protein is more efficiently imported [20]. Therefore, Djp1p could be involved in putting or keeping newly synthesised peroxisomal proteins in a translocation-competent (monomeric?) conformation. However, the mainly cytoplasmic localisation of Djp1p is also compatible with a role at the level of the targeting process. It could, for instance, stimulate the recognition of peroxisomal proteins by their cognate receptors by either acting on the PTS-containing protein or by acting on the PTS receptor. Alternatively, Hsp40/Hsp70 may mediate the various conformational changes that either the PTS-containing protein or the various import factors have to undergo during the translocation process. Despite serious efforts to identify molecular chaperones in the peroxisomal matrix that could assist either in protein assembly or in the import process itself by pulling in the matrix proteins, none have been found. A possible exception is an Hsp70 which is localised mainly to plastids and to a minor extent to glyoxysomes. This protein contains a weak PTS2 signal that is able to direct a reporter protein into peroxisomes in yeast [68].

3.4. Two pathways for peroxisomal matrix protein targeting

The isolation of peroxisome assembly mutants has resulted in the identification of a large number of genes encoding proteins (peroxins) involved in per-
oxisome biogenesis (for review see [8,13]). Phenotypic analysis of S. cerevisiae peroxisome assembly mutants revealed that import of PTS1 proteins and PTS2 proteins could be separated genetically [69]: in Δpex5 cells, PTS1 proteins were mislocalised to the cytosol and PTS2 proteins were imported into peroxisomal structures: in Δpex7 cells, import of PTS2 proteins was disturbed whereas import of PTS1 proteins was unaffected (Fig. 2). In the other pex mutants, both PTS1 and PTS2 proteins were mislocated to the cytosol, which suggested that some components of the import machinery for matrix proteins are used by both PTS1 and PTS2 proteins [69]. Similar phenotypes were observed among the fibroblasts derived from patients suffering from peroxisome biogenesis disorders [44,70]. These differential import mutants turned out to be mutated in their soluble PTS receptors. On the other hand, most pex mutants with a general import defect are mutated in either a peroxisomal integral-membrane protein or a peroxisomal membrane-associated protein.

3.5. The PTS1 receptor, Pex5p

The PTS1 receptor has been shown to be mutated in PTS1-specific import-deficient mutants in yeast (pex5) and man. Pex5p has been shown to be the PTS1 receptor by its specific interaction with the PTS1 in the two-hybrid assay, and its direct interaction with PTS1-containing peptides (for review see [35,71,72]).

The subcellular localisation of Pex5p has strong implications as to how it functions. However, this has been a matter of debate between various groups that work on different model organisms. Pex5p has been found to: (1) both peroxisome-associated and in the cytosol; (2) exclusively in the peroxisomal matrix; and (3) mainly associated with the peroxisomal membrane. The various localisations led to four speculative models of PTS1 receptor functioning (for review see [10,35,71]). Careful quantitation of the steady-state distribution of Pex5p in S. cerevisiae, P. pastoris and human fibroblasts has revealed that Pex5p is located mainly in the cytoplasm with only a few percent being peroxisome-associated [73–75]. The dual localisation of Pex5p in these experiments suggests that it functions as a cycling receptor which picks up a PTS1-containing protein in the cytosol and delivers it at the peroxisomal membrane. The rate-limiting step in Pex5p cycling might vary depending on organisms and culture conditions, thereby leading to a variable steady-state distribution between cytoplasm and association with peroxisomes. The observed intraperoxisomal pool of the PTS1 receptor might either be formed by dead-end molecules or might reflect an import pathway by which the PTS1 receptor is co-imported with the PTS1-containing protein. Since the PTS1 receptor is thought to function catalytically, it should subsequently be exported out of peroxisomes back into the cytoplasm for another round of targeting.

Experimental evidence supporting cycling of the PTS1 receptor between cytoplasm and the peroxisomal surface was obtained in human fibroblasts. Conditions that inhibit peroxisomal protein import in vitro (low temperature and ATP depletion) could partially trap Pex5p at the surface of peroxisomes in vivo [75]. Pex5p returned to the cytoplasm after restoration of import conditions. Pex5p accumulation at the peroxisomal membrane could be repeated by another incubation under protein import inhibitory conditions.

Although early studies in yeast showed that the PTS1 receptor is required for PTS1 import only, studies in mammalian cells show a requirement for the PTS1 receptor in PTS2 import [76–79]. Two isoforms of mammalian Pex5p have been identified that differ in the presence of an additional exon. This additional exon is required for PTS2 import [79,80].

3.6. The PTS2 receptor, Pex7p

The PTS2 receptor is encoded by the PEX7 gene [81–86]. ScPex7p has been shown to interact with the PTS2 in a two-hybrid assay and by co-immunoprecipitation with a PTS2-containing protein. Furthermore, a temperature-sensitive mutation in the thiolase PTS2 could be suppressed by overexpression of Pex7p in S. cerevisiae [36,87]. The localisation of yeast Pex7p has been highly debated. Based on carboxy-terminally epitope-tagged versions of ScPex7p, Pex7p has been suggested to be located mainly inside peroxisomes [82,87]. After overexpression of aminoterminally epitope-tagged Pex7p, only a minor amount of Pex7p was found associated with peroxisomes, with most residing in the cytosol [36,81].
Elgersma et al. [86] obtained similar results by overexpression and epitope tagging of *P. pastoris* Pex7p. However, when they studied the subcellular distribution of unmodified endogenous Pex7p (using a polyclonal antiserum directed against PpPex7p), they found Pex7p located mainly in the cytoplasm with only a minor fraction present inside peroxisomes. Two models have been postulated for Pex7p function. In the cycling receptor model, Pex7p picks up a PTS2-containing protein in the cytoplasm and delivers it to the peroxisomal membrane. Pex7p and ligand might either dissociate at the peroxisomal membrane with only the ligand being translocated and Pex7p returning back into the cytoplasm, or ligand and Pex7p may be co-imported into peroxisomes where they dissociate, with Pex7p being exported back into the cytoplasm. In an alternative model, Pex7p functions as an intraperoxisomal PTS2 receptor that pulls PTS2-containing proteins into the peroxisomal matrix in a manner similar to how mitochondrial Hsp70 and BiP are thought to contribute to import of proteins into mitochondria and the endoplasmic reticulum, respectively (reviewed by [88]). Interestingly, ScPex7p is able to associate with two peroxins (Pex18p and Pex21p) that are functionally redundant and that display a low amino acid sequence homology [16]. Cells lacking both Pex18p and Pex21p fail to import PTS2-containing proteins and Pex7p is located exclusively in the cytosol. These observations suggest that Pex18p/Pex21p function in directing Pex7p to peroxisomes. The localisation of Pex18p (mainly cytosolic, with a minor fraction associated with the cytoplasmic face of the peroxisomal membrane) is in agreement with such a role. For our understanding of Pex7p functioning, it is crucial to find out whether Pex18p/Pex21p target the loaded PTS2 receptor or the unloaded receptor to peroxisomes. It is interesting to note that by using the two hybrid assay, Purdue et al. [16] were able to show that Pex18p could interact with a PTS2-containing protein only in the presence of the *PEX7* gene.

### 3.7. Docking and translocation

At the peroxisomal membrane we can distinguish three events that contribute to the translocation of peroxisomal matrix proteins across the membrane. First, the PTS-containing protein complexed to its cognate receptor binds to a component of the membrane-residing docking machinery. Second, the PTS-containing protein is delivered to the translocation machinery and is translocated across the membrane. Third, the receptor shuttles back to the cytoplasm and the docking and translocation machinery can receive new substrates.

Candidate proteins that could facilitate docking and translocation were first identified in *S. cerevisiae* (for review see [7]). Both the PTS1 receptor and PTS2 receptor have been shown to interact with a peroxisomal membrane complex that consists of at least three different proteins (Fig. 2); these are either integral peroxisomal membrane proteins (Pex13p) or are associated with the cytoplasmic face of the peroxisomal membrane (Pex14p and Pex17p) [14,73,74,89–93]. Their multiple binding sites for the PTS receptors on this complex which may be redundant, although the peroxins themselves are not redundant for the import process (see below). Cells lacking either of these peroxins (Pex17p or Pex13p) fail to import both PTS1 and PTS2 proteins. Therefore, loss of one of its components could affect the whole complex, and since this heteromeric complex contains the putative docking sites for both the PTS1 receptor and the PTS2 receptor, this would lead to a block in both PTS1 and PTS2 protein targeting and import. Alternatively, the function of Pex13p, Pex14p and Pex17p may not be limited to the formation of docking sites for PTS receptors, but they may also part of the translocation machinery (translocon?). The multiple PTS-receptor-binding peroxins that are located at the peroxisomal membrane (Pex13p, Pex14p and Pex17p) might reflect the existence of an import cascade where successive interactions of a PTS receptor with peroxins at the peroxisomal membrane might induce conformational changes in the PTS receptor, which are required for: (1) docking; (2) dissociation of the PTS-containing protein; (3) passage of the PTS-containing protein to other peroxins; and (4) recycling of the receptor to the cytosol [7,90]. For the PTS2 receptor this cascade might also involve Pex18p/Pex21p in addition to the Pex13p/Pex14p/Pex17p complex.

Additional peroxins that are likely to be involved in peroxisomal matrix protein import are Pex2p and Pex12p. These integral peroxisomal membrane pro-
teins contain a RING finger domain that is thought to mediate protein–protein interactions [94]. In patient cells, in which either their PEX2 or PEX12 gene is mutated, peroxisomal matrix import is impaired and the PTS1 receptor has been shown to accumulate at the cytoplasmic face of the peroxisomal membrane [75]. It is possible that Pex2p and Pex12p are required for translocation of PTS-containing proteins and that a block at this step results in accumulation of the PTS1 receptor (possibly loaded with cargo) at the peroxisomal membrane, analogous to a block in translocation caused by ATP depletion [75]. Alternatively, Pex2p and Pex12p may be required either for passage of the PTS1 receptor through an import cascade or for recycling of the PTS1 receptor back into the cytoplasm. Since PTS2 import is also impaired in these patients’ fibroblasts, it would be interesting to study the localisation of the PTS2 receptor in these patients. The PTS1 receptor has been shown to accumulate inside peroxisomes in H. polymorpha Δpex4 mutant cells [95] and in a patient with a mutation in its PEX10 gene [75]. It is for future research to establish whether these peroxins are required for export of the PTS1 receptor back into the cytoplasm or whether they normally prevent the PTS1 receptor from entering peroxisomes.

The mechanism by which the import machinery can accommodate the translocation of folded and oligomerised peroxisomal proteins across the peroxisomal membrane remains enigmatic. One way to accommodate the translocation of folded proteins across membranes is via large pores analogous to nuclear pores. A nuclear pore can mediate the passage of large multisubunit complexes and particles up to 25 nm. In contrast to the nuclear pore, which provides a 9-nm diffusion channel through which molecules smaller than 60 kDa can diffuse, the putative peroxisomal pore should be able to open transiently to allow translocations to occur but should then close completely to maintain the permeability barrier for small molecules. Freeze-fracture electron microscopy studies have failed to identify large protein complexes in the peroxisomal membrane with a structure similar to the nuclear pore [96]. It has been postulated that this putative pore assembles only transiently and is therefore hard to detect [6]. An alternative way to import peroxisomal proteins would be via an endocytic event occurring at the peroxisomal membrane [6]. Newly synthesised peroxisomal proteins are targeted to a specific region on the surface of peroxisomes, which invaginates and buds off into the peroxisomal matrix. Subsequent degradation of the membrane or redistribution of membrane lipids will then lead to release of the cargo in the matrix. However, small internal vesicular structures are found only rarely, and whether these structures are import vesicles is not known. These two completely opposing models illustrate the gap in our knowledge concerning the actual protein translocation/incorporation event. Recently, an evolutionarily conserved pathway has been identified for transport of stably folded proteins across the chloroplast thylakoid membrane and bacterial inner membrane. Also for this pathway the translocation mechanism remains unclear (for review see [97]).

Jamming of the import site by proteins that have to be transported across other membranes (mitochondria, ER, chloroplast and bacterial plasma membrane) has been very informative for the composition and architecture of the translocation apparatus of these organelles. Unfortunately, no jamming of PTS-containing proteins in vivo has ever been shown. The recent observation that a peroxisomal fusion protein get stuck in the import site in vitro [21] might open the way to investigate the components and architecture of the peroxisomal translocation apparatus in more detail.

3.8. The peroxisomal matrix

Upon translocation of a PTS-containing protein across the peroxisomal membrane, it might either remain in the lumen of the peroxisome or associate with the inner leaflet of the peroxisomal membrane. Whereas most PTS-containing proteins are freely distributed through the peroxisomal matrix, some PTS-containing proteins, including the acyl-CoA synthetase Faa2p, are concentrated at the inner leaflet of the peroxisomal membrane [98]. Whether Faa2p associates with lipids or with a peroxisomal membrane protein is not clear.

For some proteins folding and assembly might occur in the cytoplasm whereas other proteins are imported as monomers and have to assemble inside peroxisomes. The high concentration of monomers and cofactors (for instance FAD) inside peroxisomes
may drive oligomerisation, so that no additional folding enzymes/factors are required for protein assembly. Alternatively, folding enzymes/factors may be required but these have not yet been identified. Various peroxins have been proposed to function inside peroxisomes to stimulate peroxisomal protein import. As has been discussed above, a varying fraction of the PTS receptors has been found inside peroxisome. Another peroxin that might be involved in protein translocation is Pex8p which is an intraperoxisomal protein that is associated with the inner leaflet of the peroxisomal membrane [99–101]. Its role in the import process remains to be established.

4. Peroxisomal membrane proteins

The peroxisomal membrane harbours a large set of proteins that are required for metabolite transport, matrix protein import, membrane protein insertion, segregation to progeny cells and proteins that modulate organelle shape and spacial distribution in the cell. As has been shown for peroxisomal matrix proteins, integral peroxisomal membrane proteins (PMPs) are synthesised on free polyribosomes [102–104] with one possible exception [104]. Pulse-chase experiments revealed that the rat peroxisomal ABC transporter Pmp70p is released at its mature weight into the cytosol (defined as a 100,000×g supernatant) before it binds peroxisomes. Peroxisome binding is rapidly followed by insertion into the membrane as determined by resistance to alkaline extraction [105]. These in vivo studies clearly demonstrated that Pmp70p is post-translationally imported. Furthermore, in vitro studies confirm and extend these studies. Pmp70p and the multi-membrane-spanning peroxisomal membrane protein Pmp22p bind and insert specifically into purified rat liver peroxisomal membranes [105,106]. Binding and insertion depend on the presence of both cytosolic and peroxisome-associated factors. ATP- or GTP-hydrolysis are not required in this binding and insertion assay (see below).

4.1. Targeting of peroxisomal membrane proteins

Genetic studies have revealed that PMPs are targeted/inserted (at least partly) independently from the peroxisomal matrix protein import pathways. Firstly, most pex mutants are disturbed in matrix protein import but assemble their membrane proteins properly with only a few exceptions (see below). These observations are compatible with a model in which translocation of matrix proteins and insertion of PMPs require specific components as well as common components. However, a more likely explanation is that, due to a defect in PMP insertion, matrix protein import is secondarily affected since the latter process depends on the presence PMPs (for instance Pex13p).

Secondly, the targeting signals for PMPs are distinct from the PTS1 and PTS2. The PTS for C. boivinii Pmp47p resides in a 20 amino acid hydrophilic loop between two membrane-spanning regions [107]. Similar amino acid sequences have been identified in regions of Pex3p and Pex15p that are required for membrane targeting [108–110] (see below for more detail).

4.2. Factors required for PMP insertion

Post-translational import of hydrophobic proteins requires the presence of accessory proteins that keep the protein soluble and direct it to the acceptor membrane. Some of the cytosolic and membrane factors required for binding and insertion of PMPs into peroxisomal membranes have been identified. Indeed, rat Pmp22p synthesised in a reticulocyte lysate assembles into two complexes that keep this hydrophobic protein soluble in the absence of peroxisomal membranes [111]. In the first complex, Pmp22p was found to be associated with the TCP1 ring complex, the cytosolic chaperonin. In the second complex, Pmp22p was bound to a 40-kDa polypeptide. Pmp22p present in this second complex inserted with high efficiency into peroxisomal membranes and therefore the 40-kDa protein has been postulated to be the cytoplasmic Pmp22p receptor [111]. The identity of this 40-kDa protein remains to be established.

As mentioned above, analysis of yeast and mammalian pex mutants have resulted in the identification of three peroxins required for insertion of peroxisomal membrane proteins [108,109,112,113]. These peroxins (Pex3p, Pex16p and Pex19p) might be involved in either the PMP insertion process itself.
or alternatively, in the formation of peroxisomal membranes.

There are now various observations that might indicate that not all peroxisomal PMPs are post-translationally imported into the peroxisomal membranes directly but might reach peroxisomes via the endoplasmic reticulum (for review see [11,12]). Analyses of the topogenic signals of two yeast PMPs suggest a relationship between peroxisomes and ER. The amino-terminal 40 amino acids from \textit{P. pastoris} Pex3p contain all the information for targeting and insertion into peroxisomal membranes. The first 16 amino acid residues, which precede the putative transmembrane domain, can direct a reporter protein to the ER. Whether this fusion protein resides inside the ER or whether it associates with an ER surface component has not been investigated [109]. Overexpression of wild-type and modified versions of Pex15p (truncation or fusion proteins) localise to the ER or plasma membrane [110]. These results suggest (but do not prove) that Pex3p and Pex15p are normally targeted to the ER membrane and are subsequently sorted to the peroxisomal membrane. This process might be disturbed by overexpression or truncation of these proteins. In line with this, the Pex3p membrane-spanning region would contain the ER-to-peroxisome sorting information. A more detailed analysis of this amino acid sequence will reveal whether such specific sorting signals are indeed present.

However, we should be careful about studying the targeting/sorting signals of peroxisomal membrane proteins after overexpression or truncation. For example, overexpression of ScPat1p, a yeast homologue of rat Pmp70p, results in mislocalisation to the nuclear envelope and the lateral ER. This suggests that cryptic ER targeting signals are present in some peroxisomal PMPs (E.H.H. unpublished results), or worse, that the ER constitutes a sink that attracts overproduced hydrophobic proteins.

Experimental evidence has been provided by Tito-renko and Rachubinski [114] who showed that at least two peroxins of the yeast \textit{Yarrowia lipolytica}, Pex2p and Pex16p, are targeted first to the ER, where they obtain ER-specific modifications, and are subsequently sorted to peroxisomes. Signals that mediate ER targeting and subsequent sorting to peroxisomes have not yet been identified. Despite many attempts have been made, no PMPs have been observed to reach peroxisomes via the endoplasmic reticulum in other model systems (see, for instance, [110,112,115]).

4.3. Peripheral peroxisomal membrane proteins

Several peroxisomal membrane proteins have been identified that associate with the peroxisomal membrane. Are these latter proteins specifically binding to protein determinants of the peroxisomal membrane or are these proteins recognising specific lipid determinants? Alternatively, specificity might be achieved by a combination of both, as has been described for the endosomal RING FYVE domain-containing protein EEA1 [116]. For several peripheral membrane-associated peroxins it has now been shown that they indeed interact with PMPs directly or indirectly. For instance, the farnesylated peroxin Pex19p associates with the PMP Pex3p [15] and Pex14p interacts with the PMP Pex13p [90].

A major obstacle in research of peroxisomal membrane protein studies is the lack of solid experimental techniques to verify whether proteins are integral membrane proteins or whether they are associated with the peroxisomal membrane. This has led to many contradictory results. A well-established way of addressing this question has been extraction of membranes with 0.1 M sodium carbonate, pH 11.5, followed by pelleting of membranes at 100 000 \( \times \) g. However, it seems that the outcome of this experiment is influenced by the source of the membranes [117–119]. Another major disadvantage is the lack of modifications of peroxisomal proteins on the luminal side of the organelle. Therefore, topology studies have to rely on either protease protection experiments or analysis of fusion proteins (which might influence topology), or on experiments in which antisera against (certain regions of) membrane proteins are tested for their ability to bind antigen after selective permeabilisation of the plasma membrane. This limited set of techniques combined with the use of various model systems have lead to conflicting data concerning the topology of a number of peroxins including for instance Pex2p, Pex3p, Pex11p and Pex12p. Therefore, new tools have to be developed and inter-laboratory differences have to be sorted out systematically.
5. Species-specific differences in peroxisomal protein import

Some details of the import process vary between species. As discussed above, the mammalian PTS1 receptor is required for PTS2 protein import whereas in yeast, the PTS1 receptor functions only in PTS1 protein import. H. polymorpha Pex4p is required specifically for PTS1 import whereas in other yeast species, Pex4p is required for import of both PTS1- and PTS2-containing proteins [95,120,121]. In particular, the yeast Y. lipolytica seems to be an exception with respect to both matrix and membrane protein import. For instance, Pex2p and Pex16p reach peroxisomes via the endoplasmic reticulum [114]. This has not been observed for other Pex2p and Pex16p orthologues. Furthermore, the peroxins Pex1p and Pex6p are required for both peroxisome assembly and secretion of some proteins [11]. This is in contrast to all other organisms in which pex1 and pex6 mutants have been isolated.

6. Future challenges

Although genetic and biochemical approaches have resulted in the identification of most of the components of the matrix protein import machinery, the mechanism of peroxisomal protein translocation remains unsolved. For instance, are proteins translocated through a channel or are they engulfed by peroxisomal membranes which dissolve after entering the peroxisomal lumen? Where do PTS receptors and PTS-containing proteins dissociate, in other words, are PTS receptors co-imported and subsequently exported or does dissociation take place at the cytoplasmic surface of the peroxisomal membrane? Is there a permanent or a transient docking or import complex? Furthermore, it would be rather inefficient if PTS receptors could dock at the peroxisomal membrane without being loaded with a PTS-containing protein. Therefore, it is likely that binding of ligand induces a conformational change in the PTS receptor which might lead to exposure of a domain that can interact with the docking complex. So what is that docking domain and how are conformational changes mediated?

These kind of questions need experimental approaches whereby we study the dynamics of the peroxisomal protein import process. One approach would be to use conditional alleles for the various peroxins in combination with radioactive pulse-chase experiments to study the effects on other peroxins and the import process. Additionally, we have to study the peroxins at the structural level to find out what the protein–protein interaction domains are and how binding to these domains results in conformational changes of the various peroxins. Furthermore, the promising in vitro import systems developed for plant glyoxysomes will certainly contribute to insight in the role of peroxins in the import process and the sequence of events during translocation.

The targeting and insertion of membrane proteins is less well characterised compared to import of peroxisomal matrix proteins. Novel genetic screens could identify more components for this process. The role of the endoplasmic reticulum in peroxisomal membrane protein sorting is unclear for most organisms and needs to be verified. Since most genetic screens did not select for conditional mutants, it remains possible that we have missed mutants in essential genes that might function in both peroxisome assembly and the secretory pathway or other essential processes.

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References


