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Overproduction of Pex5p Stimulates Import of Alcohol Oxidase and Dihydroxyacetone Synthase in a Hansenula polymorpha pex14 Null Mutant*

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Hansenula polymorpha Δpex14 cells are affected in peroxisomal matrix protein import and lack normal peroxisomes. Instead, they contain peroxisomal membrane remnants, which harbor a very small amount of the major peroxisomal matrix enzymes alcohol oxidase (AO) and dihydroxyacetone synthase (DHAS). The bulk of these proteins is, however, mislocated in the cytosol. Here, we show that in Δpex14 cells overproduction of the PTS1 receptor, Pex5p, leads to enhanced import of the PTS1 proteins AO and DHAS but not of the PTS2 protein amine oxidase. The import of the PTS1 protein catalase (CAT) was not stimulated by Pex5p overproduction. The difference in import behavior of AO and CAT was not related to their PTS1, since green fluorescent protein fused to the PTS1 of either AO or CAT were both not imported in Δpex14 cells overproducing Pex5p. When produced in a wild type control strain, both proteins were normally imported into peroxisomes. In Δpex14 cells overproducing Pex5p, Pex5p had a dual location and was localized in the cytosol and bound to the outer surface of the peroxisomal membrane. Our results indicate that binding of Pex5p to the peroxisomal membrane and import of certain PTS1 proteins can proceed in the absence of Pex14p.

Most organellar proteins in eukaryotic cells are synthesized on free or membrane-bound cytosolic ribosomes and sorted to their final destination by unique targeting signals. These signals are recognized by specific receptor proteins, which are either components of the cytosol (e.g. signal recognition particle for the endoplasmic reticulum) or importin α for nuclear proteins) or are bound to the membrane of the target organelle (e.g. Tom proteins at the mitochondrial outer membrane). Following recognition of the targeting signal and subsequent routing of the receptor-cargo complex to the actual import site, the protein is transported into the organelle. Generally, proteins are released from their receptors prior to import. However, in the case of nuclear protein import the receptor dissociates after transport through the nuclear pore complex, followed by export of the receptor back to the cytosol.

For peroxisomal matrix proteins comparable mechanisms may exist. Sorting of these proteins is mediated by specific targeting signals, termed PTS1 and PTS2. The PTS1 is located at the extreme carboxyl terminus of most peroxisomal matrix proteins and consists of the tripeptide SKL-COOH or a conserved variant thereof (5–7). In the methylocrophic yeast Hansenula polymorpha, the key enzymes of methanol metabolism, alcohol oxidase (AO),1 dihydroxyacetone synthase (DHAS), and catalase (CAT), all contain a PTS1 (8, 9). The PTS2, a nonapeptide with the consensus RLX₅(H/Q)L, is located at the amino terminus and observed for a limited number of matrix proteins (7, 10, 11). Typical examples of these are peroxisomal thiolases (12, 13) and H. polymorpha amine oxidase (14).

The PEX5 gene encodes the receptor for PTS1 proteins, Pex5p (15–17). Yeast PEX5 null mutants (Δpex5) are characterized by a complete block of PTS1 protein import. In such mutants the PTS2 protein import machinery is normally functioning, which indicates that both pathways can act independently in yeast. This was confirmed by the finding that in PEX7 null mutants, which lack the PTS2 receptor Pex7p, PTS2 proteins are mislocated in the cytosol, whereas PTS1 proteins are still properly imported (18, 19).

Pex5p binds the PTS1 of newly synthesized peroxisomal matrix proteins upon their synthesis in the cytosol. Subsequently, this receptor-cargo complex is thought to interact with proteins at a putative Pex5p docking site at the peroxisomal membrane (11, 20). Peroxins proposed to be involved in the docking and subsequent translocation process include Pex13p (21–23), Pex14p (24–26), and Pex17p (27). The absence of either one of these proteins results in defects in both PTS1 and PTS2 protein import. In various genetic and biochemical studies a direct interaction of Pex5p with Pex13p and Pex14p has been demonstrated (21, 22, 25, 26, 28). Moreover, interactions between Pex13p and Pex14p and between Pex14p and Pex17p have been reported (27).

Our current working model of PTS1 protein import in H. polymorpha includes that in the Pex5p-cargo complex is translocated across the peroxisomal membrane into the organellar matrix, followed by dissociation and subsequent export of Pex5p from the matrix back to the cytosol (4). This model is based on the finding that in this organism Pex5p is present both inside peroxisomes and the cytosol (17). Also, evidence has been obtained that Pex4p, a ubiquitin-conjugating enzyme, plays a crucial role in recycling of Pex5p back to the cytosol (29). This was evident from the finding that overexpression of PEX5 suppresses the PTS1 protein import defect in H. poly-

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1 The abbreviations used are: AO, alcohol oxidase; DHAS, dihydroxyacetone synthase; CAT, catalase; GFP, green fluorescent protein; WT, wild type; kb, kilobase pair.
Overproduction of Pex5p in H. polymorpha pex14 Null Mutant

**TABLE I**

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<td>pHIPZ4 containing the GFP gene fused to the tetratetrapeptide LARF</td>
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**Construction of pHIPZ4**—First the zeocin selection cassette was taken from pPIC2a (Table I) as a 1.1-kb MluI (Klenow fill-in of sticky ends)::BamHI fragment and inserted into Asp718 (Klenow fill-in of sticky ends)::BamHI-digested pBluescript II KS<sup>+</sup> (Table I), resulting in pFEM39. Subsequently, a 2.0-kb NotI-BglII fragment, containing the amino oxidase promoter region (P<sub>AOX</sub>), was taken from pHIP4 (Table I) and inserted into NotI-BglII-digested pFEM39, resulting in pHIPZ4.

**Construction of Strains Producing PTS1-containing GFP Proteins**—The chimeric gene encoding GFP fused to the targeting signal of AOX (LARF-COOH) was constructed by polymerase chain reaction-mediated amplification of the GFP gene of pEGFP-C1 (Table I) using the following oligonucleotides (oligonucleotides were obtained from Life Technologies, Inc.): upstream 5'-AGA AAG CTT ATG GTG AGC AAG GGC-3' (HI site underlined) and downstream 5'-CCC GTC GAC TTA GAA TCT GGC CAG CTT GTA CAG CTC GTC-3' (SalI site underlined) and the nucleotides that code for the tetrapeptide LARF are depicted in italics. For the construction of the gene encoding GFP fused to the targeting signal of CAT (SKI-COOH) the following primers were used: upstream 5'-CCC GGA TCC ATG AGC AAG GGC GAG-3' (HI site underlined) and downstream 5'-CCC GTC GAC TTA GAA TCT GGC CAG CTT GTA CAG CTC GTC-3' (SalI site underlined) and the nucleotides that code for the tetrapeptide SKI are depicted in italics. The overexpressed GFP under control of the amino oxidase promoter region (P<sub>AOX</sub>) was produced in H. polymorpha strains and plasmids.

**MATERIALS AND METHODS**

**Organisms and Growth Conditions**—The H. polymorpha strains used in this study are listed in Table I. H. polymorpha was grown at 37 °C in YPD medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, and 1% (w/v) glucose or in mineral medium (30) supplemented with 0.5% (w/v) glucose or 0.5% (w/v) methanol as carbon source and 0.25% (w/v) ammonium sulfate or methylamine as nitrogen source.

**Construction of Pex Mutants Overexpressing PEX5—H. polymorpha Δpex1, Δpex3, Δpex6, pex10-1, and Δpex14 mutants were transformed with SstI-linearized plasmid pHIPX4-PEX5 (Table I) using electroporation (31). Site-directed integration of the plasmids at the P<sub>AOX</sub> locus was confirmed by Southern blot analysis. Strains containing multiple copies of the pHIPX4 derivatives were selected as reported earlier (29).**
**RESULTS**

**Peroxisome Formation Is Restored in Δpex14 Cells Overproducing Pex5p**—Previously, we observed that overexpression of *PEX5* restored the formation of peroxisomes in *H. polymorpha* Δpex4 cells (29). To determine whether this phenomenon also occurs in other *H. polymorpha* pex mutants, a *PEX5* overexpression cassette (*P_{AOX}PEX5*) was integrated in two or multiple (3–5) copies into the genomes of Δpex1, Δpex3, Δpex6, Δpex10–1, and Δpex14 (Table I). Cells of these strains were incubated for 24 h in methanol-containing media to induce the *P_{AOX}* and the major peroxisomal PTS1 proteins AO and DHAS. None of the strains showed significant growth on methanol indicating that full restoration of peroxisome formation did not occur in any of the strains. We subsequently examined thin sections of KMnO₄-fixed cells from these cultures for the presence of peroxisomal structures. Methanol-grown WT cells, used as a control, characteristically contain several large peroxisomes (Fig. 1A). In Δpex1, Δpex3, Δpex6, and *pex10–1* cells overexpressing *PEX5*, such organelles were invariably absent (Fig. 1B, *pex10–1*; others not shown). However, in Δpex14:*P_{AOX}PEX5<sup>5ce</sup> cells the presence of several peroxisomes was evident. The size of these organelles was slightly reduced compared with those present in WT control cells (Fig. 1D).

In order to compare the Pex5p levels in WT, Δpex14 and Δpex14 overproducing Pex5p Western blotting experiments were performed. These experiments revealed that methanol-induced WT and Δpex14 cells contain similar levels of Pex5p. The Pex5p level was, however, significantly increased in strains containing two or multiple copies of the *P_{AOX}PEX5* overexpression cassette (Fig. 2). Densitometric scanning of blots prepared from crude extracts of methanol-grown WT cells and Δpex14:*P_{AOX}PEX5<sup>5ce</sup> cells induced for 24 h on methanol-containing media revealed that in Δpex14:*P_{AOX}PEX5<sup>5ce</sup> the amount of Pex5p was approximately 100-fold higher than in WT control cells. Much lower overproduction of Pex5p (i.e. a 20-fold increase compared with WT cells) was obtained in a Δpex14 strain containing only two copies of the *P_{AOX}PEX5* cassette (Δpex14:*P_{AOX}PEX5<sup>5ce</sup>) upon growth for 16 h on glycerol-containing medium. Examination of ultrathin sections pre-

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**Fig. 1.** Overall morphology of methanol-induced *H. polymorpha* cells to show the re-introduction of peroxisomes in Δpex14 cells, which overproduce Pex5p. In methanol-grown WT control cells normal peroxisomes are present (A). Peroxisome development is not restored in *pex10–1::P_{AOX}PEX5* cells incubated for 24 h in methanol containing media (B) (arrow, peroxisomal remnants; +, AO aggregate). Methanol-induced Δpex14 cells contain peroxisomal remnants (C) (arrow, peroxisomal remnants). However, in identically grown Δpex14:*P_{AOX}PEX5<sup>5ce</sup> cells several peroxisomes are present (D). Also, when lower Pex5p levels are obtained in Δpex14, i.e. in Δpex14:*P_{AOX}PEX5<sup>5ce</sup> grown for 16 h on glycerol, several small peroxisomes (arrow) are observed in the cells (E). Electron micrographs are taken of KMnO₄-fixed cells, poststained with uranyl acetate. The abbreviations used are as follows: M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. The marker represents 0.5 μm.

**Fig. 2.** Western blot showing the level of Pex5p in crude extracts of methanol-grown WT (lane 1), methanol-induced Δpex14 (lane 2), Δpex14:*P_{AOX}PEX5<sup>5ce</sup> (lane 3), and Δpex14:*P_{AOX}PEX5<sup>5ce</sup> (lane 4) using a-Pex5p antibodies. Compared with WT and Δpex14 extracts, Δpex14:*P_{AOX}PEX5<sup>5ce</sup> and Δpex14:*P_{AOX}PEX5<sup>5ce</sup> extracts contained enhanced amounts of Pex5p.
pared from KMnO₄-fixed cells of this culture revealed that also a 20-fold increase in Pex5p levels resulted in the formation of small peroxisomes in H. polymorpha Δpex14 (Fig. 1E).

**PTS1 Protein Import Is Stimulated by Pex5p Overproduction in Partially Restored Δpex14 Cells**—In methanol-induced H. polymorpha Δpex14 cells normal peroxisomes are absent. Instead these cells contain peroxisomal membrane remnants that contain a very minor portion of AO and DHAS, whereas the bulk of these proteins is mislocated in the cytosol (24). Immunocytochemical analysis of glycerol-grown Δpex14::PAOXPEX5mc cells revealed that the small peroxisomes that are formed in these cells contain the PTS1 proteins AO (Fig. 3A) and DHAS (not shown). Similarly, the larger organelles present in methanol-induced Δpex14::PAOXPEX5 mc cells are characterized by significant amounts of AO (not shown) and DHAS protein (Fig. 3B). Judged from the distribution of the labeling, a portion of these matrix proteins was still mislocated in the cytosol. By using antibodies against Pex3p, a peroxisomal membrane protein, labeling was obtained at the membrane of these organelles (Fig. 3C), indicating that they indeed represent peroxisomes. Remarkably, import of a third PTS1 protein, catalase (CAT), was not significantly stimulated by Pex5p overproduction (Fig. 3D). As expected, the location of the PTS2 protein amine oxidase remained cytosolic in Δpex14::PAOXPEX5 mc cells (data not shown).

The immunocytochemical data were confirmed biochemically by cell fractionation experiments. To this end post-nuclear supernatants, prepared from methanol-grown WT cells or methanol-induced Δpex14 and Δpex14::PAOXPEX5 mc cells, were subjected to sucrose density centrifugation (Fig. 4). In gradients prepared from WT cells, peroxisomes migrate to a high density corresponding to 54% sucrose (fraction 6, Fig. 4A), indicated by a distinct peak of AO and CAT activity. Also the peroxisomal membrane marker Pex3p is present in these fractions, as evident from Western blots prepared from the gradient fractions (inset in Fig. 4A). The AO and CAT activities detected in the top fractions of the gradient most likely are due to leakage of these proteins as a result of the fractionation procedure (37). In the gradient of Δpex14 cells (Fig. 4B) AO, CAT, and Pex3p are absent at high densities. Instead, AO and CAT activities are present at the top of the gradient, indicative for a cytosolic location. Pex3p migrated to a density corresponding to 37% sucrose (fraction 17, Western blot included in Fig. 4B). Previously, evidence was provided using immunocytochemistry that the peroxisomal membrane remnants, present in Δpex14 cells, contained Pex3p and very small amounts of AO and DHAS (24). Hence, these structures most likely migrated to a density corresponding to 37% sucrose (fraction 17) of the gradient prepared from Δpex14 cells. Gradients of methanol-induced Δpex14::PAOXPEX5 mc cells again displayed a prominent peak of AO activity at 54% sucrose (fraction 7, Fig. 4C); also Pex3p was localized at this position (Western blot included in Fig. 4C). However, significant amounts of AO activity, exceeding those of WT controls, were also detected in the top fractions of this gradient (fraction 18–25) most likely representing the cytosolic portion of the AO protein demonstrated by immunocytochemistry (Fig. 3A). In contrast, CAT activity (Fig. 4C) and CAT protein, as determined by Western blotting (data not shown), was only detected in the top fractions of this gradient, thus confirming the immunocytochemical data that the import of CAT was not stimulated in Δpex14::PAOXPEX5 mc cells.

To obtain additional evidence that the AO activity peak detected at 54% sucrose in the Δpex14::PAOXPEX5 mc gradient indeed represented membrane-bound AO protein and was not due to the presence of dense AO protein aggregates and/or crystallloids, these fractions were subjected to flotation centrifugation. As shown in Fig. 5, both AO activity and the peroxisomal membrane protein Pex3p floated to lower densities and comigrated in a distinct protein peak at approximately 50% sucrose, indicating that AO is indeed membrane-bound.

**Restoration of PTS1 Import Is Not Related to Variations in the PTS1**—Overproduction of Pex5p in Δpex14 cells significantly stimulated import of the PTS1 proteins AO and DHAS...
but not of CAT. Possibly, this may be related to differences in affinities of *H. polymorpha* Pex5p for the PTS1 sequences of these proteins, i.e. LARF-COOH in case of AO, NKL-COOH in case of DHAS, and SKI-COOH in case of CAT (8, 9). To analyze this possibility, we constructed chimeric genes encoding green fluorescent protein (GFP) fused to the respective targeting signals of AO and CAT (GFP.LARF and GFP.SKI, respectively). These genes were introduced in \( \Delta pex14::PAOX::PEX5^{mc} \) cells and a WT control strain, and the location of their translation products was determined. In *H. polymorpha* WT cells the two GFP fusion proteins were efficiently targeted to the peroxisomes, as indicated by the punctate patterns observed by fluorescence microscopy (data not shown) and immunocytochemistry, using specific α-GFP antibodies (Fig. 6A, GFP.LARF and GFP.SKI, not shown). However, in \( \Delta pex14::PAOX::PEX5^{mc} \) cells, producing either GFP.LARF or GFP.SKI, a diffuse fluorescence pattern was observed, indicative for a cytosolic location (data not shown). To examine whether minor amounts of the fusion proteins may
be imported into peroxisomes, their location was also determined immunocytochemically. The labeling patterns, using α-GFP antibodies, revealed that both proteins were confined to the cytosol (Fig. 6B, GFP.LARF and GFP.SKI, not shown).

Pex5p Accumulates in the Cytosol and at the Outer Surface of the Peroxisomal Membrane in Δpex14::P<sub>AOX</sub>PEX5<sup>mc</sup> Cells—In WT H. polymorpha Pex5p has a dual location and is present both in the cytosol and the peroxisomal matrix (Fig. 7B); a similar location is found in WT cells overproducing Pex5p (not shown; see Ref. 17). In immunocytochemical experiments using ultrathin sections of methanol-induced Δpex14::P<sub>AOX</sub>PEX5<sup>mc</sup> cells, the bulk of Pex5p labeling was found at the cytosol. In addition, labeling was observed at the peroxisomal membrane but never on the organelar matrix (Fig. 7A). In sucrose gradients of a post-nuclear supernatant prepared from homogenized, methanol-induced Δpex14::P<sub>AOX</sub>PEX5<sup>mc</sup> cells, Pex5p sedimented at two specific locations. Pex5p cosedimented with peroxisomal marker proteins at 54% sucrose (fraction 7) and was also detected at top of the gradient where cytosolic proteins are located (fractions 18–23; Fig. 8A). Characteristically, the cytosolic Pex5p was predominantly detected at lower molecular weight due to proteolytic degradation. Taken together, these data indicate that
Fractions were incubated in the presence of the indicated amounts (in mg/ml) of proteinase K (PK). The Western blots were decorated with antibodies against Pex5p and DHAS. Pex5p in WT peroxisomes is only degraded in the presence of Triton X-100, whereas in Δpex14 strains, peroxisomes from Δpex14::P\textsubscript{AOX}P\textsubscript{EX5\textsuperscript{mc}} cells contain enhanced amounts of Pex5p (lane 2). The amount of Pex5p is somewhat higher than in WT organelles (lane 3) compared with peroxisomes from Δpex14::P\textsubscript{AOX}P\textsubscript{EX5\textsuperscript{mc}} cells (lane 4). Peroxisomes were purified by sucrose density centrifugation. Equal amounts of protein were loaded per lane.

**Fig. 9.** Protease protection analysis using purified peroxisomes from methanol-induced WT (A) and Δpex14::P\textsubscript{AOX}P\textsubscript{EX5\textsuperscript{mc}} cells (B). Fractions were incubated in the presence of the indicated amounts (in mg/ml) of proteinase K (PK) with (+) or without (−) 0.1% Triton X-100 for 30 min at 0 °C. The Western blots were decorated with antibodies against Pex5p and DHAS. Only a very minor amount of Pex5p was protected against proteolytic degradation using 0.5 mg/ml proteinase K. In contrast, DHAS, used as a control, is relatively stable and is only partially degraded in the presence of proteinase K and Triton X-100, as is indicated by the weaker protein band observed in the samples containing both proteinase K and Triton X-100 compared with the samples lacking proteinase K (also OA is relatively stable, whereas CAT, which is more sensitive to proteases, cannot be used in this experiment). Equal portions of proteins were loaded in each experiment, except for the detection of Pex5p in WT peroxisomes, in which the protein concentration was 40-fold enhanced to facilitate Pex5p detection.

Pex5p is present in both cytosolic and peroxisomal fractions in gradients prepared from Δpex14::P\textsubscript{AOX}P\textsubscript{EX5\textsuperscript{mc}} cells.

Western blotting experiments revealed that the amounts of Pex5p in peroxisomal peak fractions of Δpex14::P\textsubscript{AOX}P\textsubscript{EX5\textsuperscript{mc}} gradients were strongly enhanced (40-fold) compared with WT controls (Fig. 8B); in these samples the levels of Pex5p, used as a control, were comparable (Fig. 8B).

In order to determine the precise location of Pex5p in peroxisomes of Δpex14::P\textsubscript{AOX}P\textsubscript{EX5\textsuperscript{mc}} cells, a protease protection assay was performed. Incubation of purified peroxisomes with proteinase K resulted in the degradation of most of the Pex5p, independent of permeabilization by Triton X-100 (Fig. 9B). Only a very minor amount of Pex5p was protected against proteolytic degradation using 0.5 mg/ml proteinase K. In control experiments, using purified peroxisomes from WT cells, all Pex5p was protected against proteinase K; degradation of the protein was only observed when the organelles were permeabilized. This confirms earlier findings that in H. polymorpha WT cells the peroxisomal fraction of Pex5p is located inside the organellar matrix (17).

Our results indicate that in Δpex14::P\textsubscript{AOX}P\textsubscript{EX5\textsuperscript{mc}} the additional Pex5p that is associated with peroxisomes (i.e. a 40-fold increase compared with WT) is predominantly associated with the outer surface of the peroxisomes, whereas a minor portion is present inside the organelles. This amount may be comparable to that observed in peroxisomes from WT cells, where all peroxisome-associated Pex5p is located inside the organelle.

**DISCUSSION**

Pex14p is a peroxisomal membrane protein and is essential for peroxisome biogenesis (24–26, 44–46). The finding that Pex14p is able to bind Pex5p and Pex7p, the specific receptors for the PTS1 and PTS2, respectively (25), has led to the suggestion that Pex14p is a central component of the peroxisomal protein translocation machinery, most likely mediating the membrane docking event of these receptors. A similar function has been postulated for Pex13p; moreover, Pex13p and Pex14p have been shown to physically interact (21–23, 47). Pex13p and Pex14p are probably part of a protein complex, which is characterized by a rather fixed stoichiometry of its constituting components for normal functioning (24, 38). Recently, a potential third component of this complex, Pex17p, has been identified (27).

Pex14p is not essential for accumulation of Pex5p at the Organellar Surface—We have shown that H. polymorpha cells, lacking Pex14p, could import specific PTS1 proteins under conditions that Pex5p was overproduced. A dose-response correlation was observed between the level of Pex5p produced and the amount of PTS1 proteins imported. A plausible explanation for restoration of import is to assume that the import of these proteins proceeds via a series of consecutive steps (comparable to matrix protein import in mitochondria for example), one of which involves the function of Pex14p (Fig. 10). Accordingly, it can be envisaged that excess amounts of Pex5p can compensate for the absence of a single component of this machinery, i.e.
Overproduction of Pex5p in H. polymorpha pex14 Null Mutant

Pex14p. The accompanying phenomenon, namely the accumulation of Pex5p at the peroxisomal membrane, could then be understood by assuming that Pex14p is in fact not the initial docking site of the Pex5p-cargo complex but functions at a later stage in the import pathway where it is essential for the efficiency of this process. Consequently, the absence of Pex14p would then strongly slow down import and could rise to the accumulation of Pex5p, blocked at the early steps of translocation (Fig. 10). It should be stressed that in WT H. polymorpha Pex5p was never found to be accumulated to the outer surface of the organelle (Fig. 7B; 17), even when PEX5 was overexpressed (29). However, in other organisms membrane-bound Pex5p has been observed (21, 23, 48). In particular, in mammalian cells enhanced amounts of Pex5p were bound to the peroxisomal surface under conditions that inhibited protein translocation (e.g. upon ATP-depletion, low temperature; Ref. 50); a similar observation was made in mutant cell lines blocked in matrix import due to mutations in PEX2 or PEX12 (49). The mechanisms, underlying these phenomena, could in fact be comparable to those made in Δpex14::P_AOX·PEX5<sub>mtc</sub> cells, namely that, dependent on the nature of the block in import, Pex5p can accumulate at the peroxisomal membrane. Remarkably, the location of Pex5p in peroxisomes of Δpex14::P_AOX·PEX5<sub>mtc</sub> cells was opposite that in Δpex4 cells, overproducing Pex5p, in that the latter cells Pex5p had accumulated at the luminal site of the peroxisomal membrane (29) (Fig. 10).

If we assume that Pex14p is not involved in the initial recruiting of Pex5p from the cytosol, this process should be fulfilled by other peroxisomal membrane proteins to explain the import in Δpex14::P_AOX·PEX5<sub>mtc</sub> cells. A possible candidate is Pex13p, but the involvement of additional proteins cannot be ruled out. An example of this could be a recently identified 140-kDa protein in peroxisomal membranes of rat liver which binds human Pex5p in ligand blots (45). However, this protein was not further analyzed.

Pex5p Overproduction in Δpex14 Cells Suppresses the Import Defect of Specific PTS1 Proteins—The finding that the peroxisomal remnants in H. polymorpha Δpex14 cells do contain trace amounts of AO protein implies that the PTS1 protein import defect in these cells is not 100% blocked (24). However, to our surprise the Pex5p-dependent import restoration in Δpex14 cells did not apply for all H. polymorpha PTS1 proteins and was only observed for AO and DHAS but not for CAT. Apparently, this difference is not related to variations in the PTS1 signal of these proteins since fusion proteins consisting of GFP fused to either the PTS1 of AO (LARF-COOH) or CAT (SKI-COOH) both were mislocalized to the cytosol of Δpex14::P_AOX·PEX5<sub>mtc</sub> cells. As shown before, in H. polymorpha WT cells the presence of a PTS1 signal at the extreme carboxyl terminus is required and sufficient to direct a reporter protein to the peroxisomal matrix, a process that involves the function of the PTS1 receptor Pex5p (8, 9, 17). However, the differential import observed in Δpex14::P_AOX·PEX5<sub>mtc</sub> cells (e.g. AO compared with GFP-LARF), overproducing Pex5p, suggests that for normal protein translocation additional factors are also involved. These factors may be related to intrinsic properties of the corresponding proteins (e.g. in case of AO and DHAS) and/or may even include yet unknown "accessory" proteins (e.g. in case of CAT). The first option, mentioned above, is not only hypothetical since evidence has been obtained that specific peroxisomal matrix proteins contain such information. A relevant example of this is that the removal of the PTS1 from Pichia pastoris AO did only result in a partial block in import of the truncated protein (50). Another indication is the finding that in two-hybrid assays the Pts1 protein carnitine acetyltransferase from Saccharomyces cerevisiae interacts with Pex5p, also when the PTS1 is removed (51). However, the finding that CAT is not imported in Δpex14::P_AOX·PEX5<sub>mtc</sub> cells lends support to the notion that the PTS1 import machinery is not uniform for all PTS1 proteins. A comparable phenomenon has been observed in case of PTS2 protein import in Yarrowia lipolytica; in this organism Pex20p is specifically involved in the import and oligomerization of the PTS2 protein thiolute (52). Other examples include Candida boidinii Pmp47 which is specifically required for import and assembly of DHAS (53) and FAD, which is essential for efficient import of AO in peroxisomes of H. polymorpha (54). Above this, variations may exist with respect to the specific requirements of import, e.g. related to the folding/oligomerization properties of imported proteins (import as monomer or oligomer), the energy requirements, or the kinetics of import. However, further experiments are required to elucidate the details of the PTS1 protein import machinery.

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