Chapter 3

Peroxisomal remnant structures in *Hansenula polymorpha* pex5 cells can develop into normal peroxisomes upon induction of the PTS2 protein amine oxidase

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Abstract

We have analyzed the properties of peroxisomal remnants in *Hansenula polymorpha* pex5 cells. In such cells PTS1 matrix protein import is fully impaired. In *H. polymorpha* pex5 cells, grown on ethanol/ammonium sulfate, conditions that repressed the PTS2 protein amine oxidase (AMO), peroxisomal structures were below the limit of detection. In methanol/ammonium sulphate-grown cells, normal peroxisomes are absent, but few small membranous structures were observed that apparently represented peroxisomal ghosts, since they contained Pex14p.

These structures were the target of a Pex10p.myc fusion protein that was produced in pex5 cells under the control of the homologous alcohol oxidase promoter (strain pex5::PAOX PEX10.MYC). Glycerol/methanol/ammonium sulphate-grown cells of this transformant were placed in fresh glucose/methylamine media, conditions that fully repress the synthesis of the Pex10p.myc fusion protein, but induce the synthesis of AMO. Two hours after the shift Pex10p.myc-containing structures were detectable that had accumulated newly synthesized AMO protein and which during further cultivation developed in normal peroxisomes. Concurrently, the remaining portion of these structures was rapidly degraded. These findings indicate that peroxisomal remnants in pex5 cells can develop into peroxisomes. Also, as for normal peroxisomes in *H. polymorpha*, apparently a minor portion of these structures actually take part in the development of these organelles.

Introduction

In eukaryotic cells most organelar proteins are synthesized in the cytosol and sorted to their target compartments by unique signals. These signals are recognized by specific receptor proteins, which are either located in the cytosol (e.g. signal recognition particle for the endoplasmic reticulum [148]) or bound to the membrane of the organelle (e.g. TOM proteins at the mitochondrial outer membrane [149]).

Proteins destined for the peroxisomal matrix are encoded by nuclear genes and synthesized in the cytosol. Specific topogenic signals have been detected at the extreme C-terminus (the PTS1) or in the N-terminus of the protein (termed PTS2) [45]. It is now widely accepted that the PTS1 and PTS2 are recognized in the cytosol by their specific receptor molecules, Pex5p and Pex7p, and that this interaction in fact initiates the actual protein import process. Both pathways converge at the docking site, consisting of at least Pex13p, Pex14p and Pex17p. Maintenance of the correct stoichiometry of the components of the docking complex seems to be important since relatively minor alterations in the
level of Pex14p already affect normal import [43]. Another peroxin complex, consisting of the two Zn-binding proteins Pex10p and Pex12p most likely binds Pex5p at a later stage after docking [49, 150]. It can be envisaged that both complexes reflect an import cascade with different affinities for the receptor/cargo complex. These interactions could lead to the dissociation of the cargo from the receptor and the handover of the cargo molecule to the translocation machinery. Alternately, the receptor/cargo complex may be completely translocated across the peroxisomal membrane, followed by dissociation of the complex in the peroxisomal lumen [36]. Indeed, in Hansenula polymorpha we have obtained evidence for this pathway, in which Pex4p, an ubiquitin-conjugating enzyme, plays a role [35].

In yeast, deletion of the PEX5 gene results in a general block in PTS1 matrix protein import. However, at PTS2 protein inducing conditions (e.g. olate, in case of H. polymorpha primary amines), small peroxisomes were observed in these mutants since both the PTS2 import and the insertion of peroxisomal membrane proteins are independent of Pex5p function [151, 152]. When H. polymorpha pex5 mutant cells were grown in carbon-limited chemostats on glucose/choline, conditions that strongly induce peroxisome proliferation, a few small peroxisomes were observed. These organelles contained the PTS2 protein amine oxidase (AMO, induced by choline), while the PTS1 proteins alcohol oxidase (AO), dihydroxyacetone synthase (DHAS) and catalase (CAT) were mislocalized in the cytosol [33]. However, under AMO-repressing conditions during growth of cells on methanol/ammonium sulphate, peroxisomes are fully absent in pex5 cells [153]. Here we analyzed whether such cells contained peroxisomal membrane structures and normal peroxisomal membrane proteins. We have used the integral peroxisomal membrane protein Pex10p, carrying a C-terminal myc tag (Pex10p.myc) as a marker protein for the detection of these structures. Also, we studied whether peroxisome development upon induction of AMO protein initiates from such structures or whether alternative mechanism existed. The results of these studies are the contents of this paper.

Materials and methods

Organisms and growth conditions

The Hansenula polymorpha strains, used in this study, are listed in Table I. The cells were cultivated in mineral media [135], containing 0.5 % (w/v) glucose, 0.5 % (v/v) ethanol or a mixture of 0.15 % (v/v) glycerol and 0.2 % (v/v) methanol as carbon sources, supplemented with 0.25 % (w/v) ammonium sulphate, 0.25 % (w/v) ethylamine, or 0.25 % (w/v) methylamine as respective nitrogen sources, at 37 °C. For the selection of mutants solid YND media were used, containing 2 % (w/v) yeast nitrogen base (Difco) and 1.5 % (w/v) agar.

Construction of H. polymorpha WT and pex5 strains, producing Pex10p.myc

A leucine-auxotrophic PEX5 deletion strain, suitable for transformation with ScLEU2-based plasmids [154], was constructed as follows. By PCR, a BamHI site was introduced downstream the 5' untranslated region (UTR) of PEX5, using the primer 5'-GGG GGA TCC ATT GAT GGT TTG TGC TCA AG-3' (BamHI-site underlined). A 2.2-kb BglII (sticky-ends filled-in using Klenow enzyme)-BamHI DNA-fragment, containing the HpURA3 gene [155] was inserted in between the engineered BamHI site and the genomic NruI site at position +1504 in the PEX5-ORF. Using BsiWI and NaeI, a PEX5 disruption cassette was obtained, containing the HpURA3 gene [155] was inserted in between the engineered BamHI site and the genomic NruI site at position +1504 in the PEX5-ORF. Using BsiWI and NaeI, a PEX5 disruption cassette was obtained, containing the HpURA3 gene [155] was inserted in between the engineered BamHI site and the genomic NruI site at position +1504 in the PEX5-ORF. Using BsiWI and NaeI, a PEX5 disruption cassette was obtained, containing the HpURA3 gene [155] was inserted in between the engineered BamHI site and the genomic NruI site at position +1504 in the PEX5-ORF. Using BsiWI and NaeI, a PEX5 disruption cassette was obtained, containing the HpURA3 gene [155] was inserted in between the engineered BamHI site and the genomic NruI site at position +1504 in the PEX5-ORF. Using BsiWI and NaeI, a PEX5 disruption cassette was obtained, containing the HpURA3 gene [155] was inserted in between the engineered BamHI site and the genomic NruI site at position +1504 in the PEX5-ORF. Using BsiWI and NaeI, a PEX5 disruption cassette was obtained, containing the HpURA3 gene [155] was inserted in between the engineered BamHI site and the genomic NruI site at position +1504 in the PEX5-ORF. Using BsiWI and NaeI, a PEX5 disruption cassette was obtained, containing the HpURA3 gene [155] was inserted in between the engineered BamHI site and the genomic NruI site at position +1504 in the PEX5-ORF. Using BsiWI and NaeI, a PEX5 disruption cassette was obtained, containing the HpURA3 gene [155] was inserted in between the engineered BamHI site and the genomic NruI site at position +1504 in the PEX5-ORF. Using BsiWI and NaeI, a PEX5 disruption cassette was obtained, containing the HpURA3 gene [155] was inserted in between the engineered BamHI site and the genomic NruI site at position +1504 in the PEX5-ORF. Using BsiWI and NaeI, a PEX5 disruption cassette was obtained, containing the HpURA3 gene [155] was inserted in between the engineered BamHI site and the genomic NruI site at position +1504 in the PEX5-ORF. Using BsiWI and NaeI, a PEX5 disruption cassette was obtained, containing the HpURA3 gene [155] was inserted in between the engineered BamHI site and the genomic NruI site at position +1504 in the PEX5-ORF.
Table I. *Hansenula polymorpha* strains and plasmids

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<th>Strains/plasmids</th>
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<td><em>H. polymorpha</em> strains</td>
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<tr>
<td>NCYC 495</td>
<td>wildtype (<em>WT</em>), <em>leu1.1</em></td>
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<td><em>pex10-1</em></td>
<td><em>pex10-1</em> mutant, <em>leu1.1</em></td>
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<td><em>pex10-1</em> + <em>P</em> _AOX_PEX10.MYC*</td>
<td><em>pex10-1</em> containing the plasmid <em>pHIPX4-PEX10.MYC</em></td>
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<td><em>PEX5</em> deletion strain, <em>leu1.1</em></td>
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<td><em>pex5:</em> + <em>P</em> _AOX_PEX10.MYC*</td>
<td><em>pex5</em> with two-copy integration of plasmid <em>pHIPX4-PEX10.MYC</em> at the <em>P</em> <em>AOX</em> locus</td>
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<td>Plasmids</td>
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<tr>
<td>pBluescript II KS⁺</td>
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<td>Stratagene, La Jolla, CA</td>
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<tr>
<td>pET4</td>
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<td>[157]</td>
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<td>pFAS01</td>
<td><em>pBluescript II KS⁺</em> containing the <em>BamH1</em>-<em>EcoR1</em> fragment of the <em>PEX10.MYC</em> PCR-product</td>
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<td>pHIPX4-PEX10.MYC</td>
<td><em>pHIPX4</em> containing the <em>BamH1</em>-<em>XhoI</em> <em>PEX10.MYC</em> fragment of pFAS01</td>
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...showed the expected genomic changes after Southern blot analysis.

The chimeric gene, encoding Pex10p.myc, was constructed by PCR-mediated amplification of the *PEX10* gene of pET4 (Table I) using the following primers (oligonucleotides were obtained from Life Technologies BV, Breda, The Netherlands): upstream 5'-AGA GGA TCC ATG TTT AAG CTT TTG TC-3' (*BamH1*-site underlined) and downstream 5'-AGA GAA TTC TTA CAA GTC TTC CTC AGA AAT AAG CTT CTC CTC TCG TAG AGG CAA CAG CTG-3' (*EcoR1*-site underlined, the nucleotides coding for the myc-epitope [158] are depicted in italics). The PCR product was ligated as a 0.94 kb *BamH1*-*EcoR1* fragment in pBluescript II KS⁺, digested with *BamH1* and *EcoR1*. The resulting plasmid, pFAS01, was digested with *BamH1* and *XhoI* and the 0.94 kb fragment was inserted into *BamH1*-*SalI* digested pHIPX4 (Table I) under control of the alcohol oxidase promoter, resulting in plasmid *pHIPX4-PEX10.MYC*. The plasmid *pHIPX4-PEX10.MYC* was introduced into a *H. polymorpha pex10-1* mutant strain by electroporation [159] to check the functionality of the hybrid protein. Also it was linearized with *StuI* and integrated at the *P* _AOX_ locus of *H. polymorpha pex5* strain [159]. Transformants were selected on YND plates and site-directed integration of the plasmid at the *P* _AOX_ locus was confirmed by Southern blot analysis. *Pex5* strains containing two copies of the *pHIPX4* derivatives were selected for further studies.

Biochemical methods

Crude extracts of *H. polymorpha* cells were prepared as detailed by Baerends *et al.* [99]. Cell fractionation procedures [54] and cytochrome c oxidase activities measurements [160] was performed as described. Protein concentrations were determined using the Biorad Protein Assay system (Biorad GmbH, Munich, Germany) using BSA as a standard. SDS-PAGE [138] was carried out as described and gels were subjected to Western blotting [137].
Fig. 1. Demonstration of the presence of peroxisomal remnants and peroxisomes containing AMO in *H. polymorpha pex5* cells. (A) Overall morphology of ethanol/ammonium sulphate-grown *H. polymorpha pex5* cells. In these cells no peroxisomal structures are visible. After 60 min of cultivation on ethanol/ethylamine a small peroxisome was observed (B). (C) Overall morphology of methanol/ammonium sulphate-induced *pex5* cells that contain few small peroxisomal remnants (arrow = peroxisomal remnants), characterized by the presence of Pex14p, as was evident upon labeling using α-Pex14p antibodies (C: inset). On glycerol/methanol/methylamine the cells contained few peroxisomes (arrow) which were formed within 2 hours (D). Using specific α-AMO antibodies, these structures were labeled (D: inset). Figs A-D: KMnO₄-fixation, insets: glutaraldehyde-fixation. The bars in (A-D) represent 0.5 μm and in the insets: 0.25 μm. Abbreviations: M-mitochondrion; N-nucleus; P-peroxisome; V-vacuole.
Peroxisomal remnant structures in pex5 cells

Fig. 2. Western blot analysis of crude extracts prepared from H. polymorpha WT and pex5 cells cultivated at various conditions. Cells were grown on glucose/ammonium sulphate (GA), ethanol/ammonium sulphate (EA), ethanol/ethylamine (EE), glycerol/methanol/ammonium sulphate (GMA) and glycerol/methanol/methylamine (GMM). The levels of the peroxisomal membrane proteins Pex3p and Pex14p were determined by decorating the Western blots with specific antibodies against these proteins. Glucose-grown cells contained minor amounts of these proteins. Upon growth on ethanol-containing medium the level of Pex3p was comparable to glucose-grown cells, but Pex14p levels were enhanced. Both proteins were strongly induced in cells grown on glycerol/methanol. The protein band indicated by the asterisk on the Western blots decorated with Pex3p antibodies represents an aspecific mitochondrial protein [161].

Nitrocellulose blots were decorated using specific polyclonal antibodies against various H. polymorpha proteins.

Electron microscopy

Cells were fixed and prepared for electron microscopy as described previously [52]. Immunolabeling was performed on ultrathin sections of unicryl-embedded cells, using specific antibodies against various proteins and gold-conjugated goat-anti-rabbit (GAR), or goat-anti-mouse (GAM) antibodies [52].

Results

Characterization of peroxisomal structures in H. polymorpha pex5 cells

The presence of putative peroxisomal membrane structures in H. polymorpha pex5 cells was first determined in cells, grown at moderate peroxisome-induction conditions on ethanol/ammonium sulphate. Under these conditions WT cells contain few organelles, characterized by the presence of isocitrate lyase and malate synthase, key enzymes of the glyoxylate cycle. Electron microscopic analysis showed that in such pex5 cells peroxisomal structures were not detectable despite extensive research also including analysis of serial sections (Fig. 1A). This was remarkable since the levels of the membrane protein Pex14p was equal to WT control cells and over 5-fold enhanced, compared to cells grown on glucose (Fig. 2). Pex3p was also detectable but not enhanced compared to glucose-grown WT cells, independent whether ammonium sulphate or ethylamine was used as the nitrogen source (Fig. 2). Ethylamine metabolism is mediated by amine oxidase (AMO), a PTS2 protein of H. polymorpha. At the morphological level, however, replacement of ammonium sulphate by ethylamine led to the rapid development of peroxisomes in ethanol-grown pex5 cells. Typically, one - or very few - peroxisomes developed within 1 hour of cultivation in the new ethylamine environment (Fig. 1B).
As expected, these organelles were characterized by the presence of AMO protein. This raises the question of the origin of these new organelles. A reason for the virtual absence of peroxisomal remnants in ethanol/ammonium sulphate-grown cells could be that these structures were below the limit of detection of electron microscopy. To enhance the level of peroxisomal proteins, pex5 cells were subsequently grown on glycerol/methanol/ammonium sulphate. Since H. polymorpha pex strains cannot grow on methanol alone [162], glycerol was applied as the growth substrate using methanol as additional energy source and peroxisome-inducer. In this way growth conditions are created which are similar for both pex5 and WT control cells, at least until the mid-exponential growth phase. WT cells grown under these conditions contain several large peroxisomes [128]. Western blot analysis revealed that the levels of Pex3p and Pex14p in glycerol/methanol-grown pex5 and WT cells were comparable, but strongly enhanced compared to the levels in ethanol- or glucose-grown cells (Fig. 2). This indicated that deletion of the PEX5 gene did not affect the amounts of peroxisomal membrane proteins in the cells.

Electron microscopic analysis of glycerol/methanol/ammonium sulphate grown pex5 cells revealed the presence of a cluster of membranous structures (Fig. 1C). These structures apparently represented peroxisomal membrane remnants, because they could be specifically labeled in immunocytochemical experiments using antibodies against the peroxisomal membrane protein Pex14p (Fig. 1C, inset). However, they did not contain alcohol oxidase, dihydroxyacetone synthase and catalase, all PTS1 proteins and key enzymes of methanol metabolism. In medium with methylamine as sole nitrogen source instead of ammonium sulphate, the pex5 cells contained distinct peroxisomes, characterized by the presence of the PTS2 protein AMO protein (Fig. 1D). This clear-cut morphological phenotype allowed us to analyze the fate of the membrane remnants during the adaptation of cells, pre-grown on glycerol/methanol/ammonium sulphate, to glycerol/methanol/methylamine-containing media in order to resolve whether these structures take part in the formation of new AMO containing peroxisomes or whether alternative mechanisms exist.

**Labeling of the peroxisomal membrane structures in pex5 cells with Pex10p.myc**

*Hansenula polymorpha* has the advantage over other yeast that PTS2 proteins are repressed or below the level of detection in cells grown on glucose, glycerol or methanol in the presence of ammonium sulphate. Previous data showed that the PTS2 import machinery is induced by primary amines, used as sole nitrogen source [163]. We have taken advantage of these properties in our studies to determine whether the peroxisomal membrane remnants can develop into peroxisomes upon induction of AMO protein by (m)ethylamine.

The second crucial condition to conduct the above experiments is the possibility to specifically tag these membrane structures. To achieve this, a hybrid gene was constructed which encoded a C-terminal myc tagged version of the peroxisomal membrane protein Pex10p (Pex10p.myc) and was placed under control of the alcohol oxidase promoter (P_{AOX}PEX10.MYC). This gene was selected since it is known that overproduction of Pex10p does not affect peroxisome integrity and protein import competence [157]. Also, Pex10p.myc has been shown to normally function in man [164].

To test the function of Pex10p.myc in *H. polymorpha*, we introduced the PEX10.MYC expression cassette in the original *H. polymorpha* pex10-1 mutant [157]. The resulting transformant grew on methanol at WT rates (data not shown). This suggested that Pex10p.myc functionally complemented the peroxisomal defect in pex10-1 cells, since any defect in
Peroxisomal remnant structures in *pex5* cells

Fig. 3. Immunocytochemical analysis of glycerol/methanol/ammonium sulphate-grown *H. polymorpha* *pex5* cells containing the
\( P_{\text{AOX}} \text{PEX10.MYC} \) expression cassette. The cells contained few small membrane structures (A, indicated by the arrow), containing Pex10p.myc (B; \( \alpha \)-myc antibodies). Pex10p is also present in these structures, as was evident upon labeling experiments using \( \alpha \)-Pex10p antibodies (C). Fig. 3A: KMnO\(_4\) fixation; Fig. 3B and 3C: glutaraldehyde-fixed cells. Bar in Fig. 3A: 0.5 \( \mu \)m. Bars in Figs. B and C: 0.25 \( \mu \)m. Abbreviations: M-mitochondrion; N-nucleus; V-vacuole.

PTS1 (and thus AO) import would prevent growth of cells on methanol [162]. This was confirmed by electron microscopic analysis, which revealed that methanol-grown cells of this transformant contained normal peroxisomes. Moreover, immunocytochemistry showed that the location of myc protein was confined to peroxisomal membranes, confirming that indeed Pex10p.myc was normally sorted to the peroxisomal membrane (not shown).

Next, we introduced the \( P_{\text{AOX}} \text{PEX10.MYC} \) cassette in the *H. polymorpha* *pex5* strain. A transformant with two copies of \( P_{\text{AOX}} \text{PEX10.MYC} \) integrated in the genome was selected and cultivated in glycerol/methanol/ammonium sulphate-containing media to induce Pex10p.myc and peroxisomal protein synthesis. Electron microscopy demonstrated that these cells contained clusters of membranous structures (Fig. 3A) that behaved like normal cell constituents in that they were donated to new developing buds (not shown; compare Fig. 9) These structures were the sole sites of gold labeling in immunocytochemical experiments, using \( \alpha \)-myc antibodies (Fig. 3B). Similar results were obtained when \( \alpha \)-Pex3p, \( \alpha \)-Pex10p, and \( \alpha \)-Pex14p antibodies were used (shown for Pex10p; Fig. 3C), indicating that these structures represent peroxisomal membrane structures. Analysis of series of consecutive sections revealed that these clusters were invariably associated with the nucleus and generally include over 20 small vesicles (Fig. 4). Taken together, these data imply that the Pex10p.myc marker protein had specifically accumulated at the peroxisomal membrane remnants in glycerol/methanol/ammonium sulphate-grown *pex5* cells (Fig. 1C) and thus, is a suitable component to tag these structures.

The co-localization of Pex10p.myc with the peroxisomal membrane remnants in *pex5::P_{\text{AOX}} \text{PEX10.MYC}* cells was confirmed by cell fractionation experiments. A post-nuclear supernatant (PNS), prepared from homogenized protoplasts of glycerol/methanol/ammonium sulphate-grown cells of this strain was subjected to sucrose density centrifugation [34].

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**Fig. 3** Immunocytochemical analysis of glycerol/methanol/ammonium sulphate-grown *H. polymorpha* *pex5* cells containing the
\( P_{\text{AOX}} \text{PEX10.MYC} \) expression cassette. The cells contained few small membrane structures (A, indicated by the arrow), containing Pex10p.myc (B; \( \alpha \)-myc antibodies). Pex10p is also present in these structures, as was evident upon labeling experiments using \( \alpha \)-Pex10p antibodies (C). Fig. 3A: KMnO\(_4\) fixation; Fig. 3B and 3C: glutaraldehyde-fixed cells. Bar in Fig. 3A: 0.5 \( \mu \)m. Bars in Figs. B and C: 0.25 \( \mu \)m. Abbreviations: M-mitochondrion; N-nucleus; V-vacuole.
Fig. 4. Six consecutive sections out of a series of a glycerol/methanol/ammonium sulphate-grown 
*pex5*:PAOX*PEX10.MYC* cell (A-F; KMnO₄-fixation). These sections clearly demonstrate the vesicular nature of 
peroxisomal membrane remnants in this strain. In Fig. 4C three structures are selected (white box) that actually 
represent vesicles, since in the previous (B) and the following section (D) of the cell only small fragments are 
present. These selected peroxisomal remnants are not detected in other sections of the cell. In addition, in Fig. 
4A and B relatively small membrane structures, compared to other peroxisomal vesicles, are detected (indicated 
by an arrow). Because of their small size, they may represent newly synthesized peroxisomal membrane 
remnants. Remarkably, the location of these small structures seems to be restricted to a small area (frequently in close association with a mitochondrion (A)). The bar represents 0.5 μm. Abbreviations: M-mitochondrion; N-nucleus; V-vacuole.

Analysis of the various fractions obtained indicated that bulk of the cellular protein was located at the top of the gradient, representing soluble, cytosolic proteins. Western blot analysis of these fractions revealed that Pex10p.myc migrated to a density corresponding to approximately 35 % (w/w) sucrose (Fig. 5B, fraction 16-18). A similar distribution pattern was observed for Pex14p. At 53 % (w/w) sucrose, where intact peroxisomes of *H. polymorpha* wild type cells normally sediment [34, 35], Pex10p.myc and Pex14p were not detectable. The protein peak at approximately 42 % (w/w) sucrose contains mitochondria judged from the distribution of the mitochondrial marker enzyme cytochrome c oxidase. The minor cytochrome c oxidase peak, present at 30 % (w/w) sucrose, most probably reflects fragmented and subsequently sealed protoplasts that were still present in the PNS. From this we conclude that the peroxisomal membrane structures present in glycerol/methanol/ammonium sulphate-grown *H. polymorpha* *pex5*:PAOX*PEX10.MYC* cells sediment to a density of 35 % (w/w) sucrose. These findings are in line with earlier data which revealed that peroxisomal remnants from other *H. polymorpha* *pex* mutants defective in matrix protein import also sedimented to relatively low densities in sucrose gradients (e.g. *pex4* [35], *pex8* [161] and *pex14* [34]).
A minor number of the peroxisomal membrane structures accumulate newly synthesized AMO to develop into normal peroxisomes

The approach to analyze whether the peroxisomal remnants in pex5::P_AOX-PEX10.MYC cells can develop into normal peroxisomes includes the initial induction of these structures by methanol, followed by the synthesis of the peroxisomal PTS2 protein AMO under conditions that the synthesis of Pex10p.myc is fully repressed (by glucose). Pex5::P_AOXPEX10.MYC cells were pre-cultivated on glycerol/methanol/ammonium sulphate to induce the synthesis of Pex10p.myc containing membrane remnants. These cells were harvested by centrifugation and incubated for 30 min at 37 °C in mineral medium, lacking the C- and N-source to remove any Pex10p.myc...
Fig. 6. Western blots prepared from crude extracts of methanol-grown *pex5::PAOX PEX10.MYC* cells shifted to glucose/methylamine- (A) and glucose/ammonium sulphate-containing media (B). Pex10p.myc was determined using α-myc antibodies. Upon the shift of cells to glucose (A and B) as sole carbon source, a rapid decrease of Pex10p.myc was observed. A similar decrease was observed for Pex3p, using α-Pex3p antibodies (A and B), indicating that the peroxisomal membrane remnants were degraded. The synthesis of AMO protein is induced after replacing ammonium by methylamine as sole nitrogen source (A). The asterix marks the α-specific protein band, using α-Pex3p antibodies (see Fig. 5). Equal volumes were loaded per lane.

Subsequently, methylamine (as N-source) was added to this medium to induce AMO synthesis together with glucose (as C-source) to concurrently block the synthesis of Pex10p.myc. At various time points after addition of these substrates cells were harvested and analyzed. The protein patterns of AMO, Pex10p.myc and Pex3p were determined by Western blotting (Fig. 6). As evident in Fig. 6A, AMO was rapidly induced within 2 hours after the shift to glucose/methylamine medium. Identical blots, decorated with antibodies against the myc-epitope, revealed a drastic decrease in Pex10p.myc levels in time. Within 2 hours after the shift the level of Pex10p-myc had decreased to approximately 20% of the level in the inoculum cells. A similar reduction was observed for Pex3p (Fig. 6A).

To analyze the initial events of peroxisome formation, a post nuclear supernatant (PNS) of glycerol/methanol/ammonium sulphate-grown *pex5::PAOX-PEX10.MYC* cells, transferred for 2 hours to glucose/methylamine media, was subjected to sucrose density centrifugation. Analysis of the various fractions obtained did not allow the unequivocal demonstration of the myc-epitope, most likely due to the diminished levels of Pex10p.myc protein. For this reason we tried to enrich the peroxisomal fraction by conventional differential fractionation of the PNS. Western analysis revealed that Pex10p.myc pelleted in the 30,000 x g (P3) organellar fraction (Fig. 7, inset). Next, this Pex10p.myc enriched organellar fraction was subjected to sucrose density centrifugation (Fig. 7). The major protein peak present in this gradient (Fig. 7A, fractions 12-13) represents mitochondria, judged from the distribution of cytochrome c oxidase activity. Western blot analysis of the various fractions collected from the gradient revealed that bulk of the
Fig. 7. Sucrose gradient, prepared from an organellar pellet (P3) obtained from homogenized, glycerol/methanol/ammonium sulphate-induced pex5::P<sub>AOX</sub>PEX10.MYC cells shifted to glucose/methylamine-containing medium (A), showing the distribution of the protein (o) in mg.ml<sup>-1</sup>, sucrose (+) expressed as percentages (w/w), and the activity of the mitochondrial enzyme cytochrome c oxidase (▲) expressed as percentages of the activity in the peak fraction, which was arbitrarily set at 100. After differential centrifugation of the PNS Pex10p.myc is present in the 30,000 x g pellet (inset). P3 and S3 designate the 30,000 x g pellet and supernatant respectively, while P4 and S4 represents the 100,000 x g pellet and supernatant respectively. Fig. 7B: Western blots analysis of the sucrose gradient to demonstrate the distribution of the myc epitope, Pex14p and AMO protein. Pex10p.myc was detected in fraction 8-10 corresponding to a density of approximately 48 % (w/w) sucrose. The peak of Pex14p colocalized with Pex10p.myc. Bulk of the AMO protein was detected in fraction 8-11. Equal volumes were loaded per lane.
Fig. 8. Morphological analysis of glycerol/methanol/ammonium sulphate-grown \textit{pex}5::\textit{P}_{\texttt{AOX}}\textit{PEX10.MYC} cells after the shift to glucose/methylamine-containing medium. Before the shift, these cells contained few small peroxisomal membranes, indicated by the arrow (A). After incubation for 2 hours on glucose/methylamine, these membrane structures could still be detected (C), although a small portion were surrounded by additional membrane layers and degraded by the vacuole (B, indicated by the arrow). Labeling of ultrathin sections of these cells using specific antibodies against the myc-epitope, showed that the peroxisomal membrane structures contained \textit{Pex10p.myc} (C, inset). After a longer incubation period (4 hours), a few small peroxisomes could be observed (D), which contained the PTS2 protein \textit{AMO}, as is evident from the immunolabeling using specific antibodies against this protein (D, inset). The electron micrographs (A-D) are from KMnO$_4$-fixed cells and the insets of glutaraldehyde-fixed cells. The bars in the insets of (C) and (D) represent 0.25 μm.
Pex10p.myc had migrated to a density corresponding to approximately 48 % (w/w) sucrose (Fig. 7B: fraction 9-10). The major portion of the peroxisomal membrane protein Pex14p was found in the same fractions. Hence, these peroxisomal proteins migrated to higher densities than the peroxisomal remnants of methanol-induced pex5::P\textsubscript{AOX}PEX10.MYC cells (approximately 35 % (w/w) sucrose, Fig. 5), but not yet to the density of mature peroxisomes of WT methanol-grown H. polymorpha cells (approximately 53 % (w/w) sucrose; [34, 35]). This location most probably reflects the relatively smaller size of the organelles, compared to WT peroxisomes. Bulk of the AMO protein co-fractionated with Pex10p.myc and Pex14p in fraction 9-10, suggesting that AMO protein may be present in the same compartment as Pex10p.myc and Pex14p. Interestingly, a portion of Pex10p.myc was also found in fraction 8, which contained only a little amount of Pex14p. Hence these fractions might contain a slightly different type of structures. In addition, almost no Pex10p.myc was detected at lower densities (fractions 11-12), which did however contain Pex14p and AMO. Possibly these fractions represent very small, newly formed peroxisomes.

The morphological adaptations of pex5::P\textsubscript{AOX}PEX10.MYC cells, shifted from glycerol/methanol/ammonium sulphate into fresh glucose/methylamine media were analyzed by electron microscopy (Fig. 8). After 2 hours, the fraction of peroxisomal membrane remnants had strongly reduced. Peroxisomal membrane structures could still be detected (Fig. 8B and C), but were frequently seen surrounded by additional layers of membranes, which is a typical feature for vacuolar uptake of peroxisomes to be degraded (Fig. 8B).

Immunocytochemistry demonstrated that Pex10p.myc was still detectable on the remaining membranous structures (Fig. 8C, inset). After 4 hours of incubation on glucose/methylamine, few small peroxisomes could be observed (Fig. 8D), which contained the PTS2 protein AMO, as was evident from labeling experiments using specific α-AMO antibodies (Fig. 8D, inset). Apart from these peroxisomes, membrane remnant structures were no longer detectable. Remarkably, the number of growing organelles was low compared to the number of vesicles originally present in the cells. Also, after a shift of glycerol/methanol/ammonium sulphate to glycerol/methanol/methylamine (thus only changing the nitrogen source), only few (up to maximally 5-6) organelles developed (data not shown).

Double labeling of ultrathin sections of the cells using α-myc and α-AMO antibodies revealed that Pex10p.myc and AMO protein co-localized on the peroxisomal structures (Fig. 9). This strongly suggests that the peroxisomal membrane remnants in glycerol/methanol/ammonium sulphate grown pex5::P\textsubscript{AOX}PEX10.MYC cells had accumulated newly synthesized AMO.

The biochemical and morphological observations indicate that our shift experiment probably had initiated two opposite processes that occurred simultaneously: glucose-induced degradation of peroxisomal membrane remnants [109] and peroxisome development due to synthesis and import of newly induced AMO protein [6]. To analyze the glucose-induced degradation process separately from the import process of AMO, glycerol/methanol-induced pex5::P\textsubscript{AOX}PEX10.MYC cells were shifted to glucose/ammonium sulphate-containing media. Crude extracts were prepared at various time points after the shift and analyzed. The protein patterns of Pex10p.myc and Pex3p were determined by Western blotting experiments (Fig. 6B). These data indicated that the levels of both proteins rapidly decreased in time (Fig. 6B), indicating that the Pex10p.myc tagged peroxisomal membrane remnants are subject to degradation under these conditions. This degradation process resembles the selective autophagy that has been described for peroxisomal remnants in
Fig. 9. Immunocytochemical demonstration that the pre-existing peroxisomal remnants containing Pex10p.myc are the target for newly synthesized AMO. Glycerol/methanol/ammonium sulphate-grown pex5::PAOX PEX10.MYC cells were transferred to glucose/methylamine medium and cultivated for 2 hours. A double labeling experiment using antibodies against myc (10 nm gold-conjugated GAM) and AMO (5 nm gold-conjugated GAR) showed that Pex10p.myc and AMO colocalized in the peroxisomal remnants (arrow = peroxisomal remnants). A small portion of these peroxisomal remnants was transferred to the bud of this cell. A more detailed image of the colocalization of Pex10p.myc and AMO is provided by the inset (the arrow indicates the 5 nm gold-conjugated GAR). Electron micrographs are taken of glutaraldehyde-fixed cells, poststained with uranylacetate. The bar in (A) represents 0.5 μm and in the inset 0.25 μm. Abbreviations: M=mitochondrion; N=nucleus; V=vacuole.

other *H. polymorpha* pex mutants [96].

Notably, in glucose/methylamine media the Pex10p.myc and Pex3p reduction is slightly less than in glucose/ammonium sulphate cultures lending support to the view that indeed fraction of these membranes takes part in new peroxisome development. Another indication for this is the morphological observation that in other *H. polymorpha* pex deletion strains re-introduction of the corresponding gene invariably results in the development of - or infrequently few - new small peroxisomes in the vicinity of the cell wall.

Discussion

In this paper we provide evidence that peroxisomal remnants present in methanol-induced *H. polymorpha* pex5 cells may develop into normal peroxisomes upon the synthesis of the key enzyme in amine metabolism, the PTS2 protein amine oxidase (AMO). The generally accepted view is that peroxisomes develop from already existing organelles by growth and fission [20]. Our data indicate that this rule can now be extended in that peroxisomes may also develop from remaining residual structures in pex5 mutant cells. Whether this is also true for the other pex mutants, e.g. after re-introduction of the defective gene, is not yet clear. We have demonstrated that the peroxisomal
remnants (‘ghosts’) in *H. polymorpha pex4* and *pex14* cells can develop into normal peroxisomes upon overexpression of the *PEX5* gene [34, 35]. Peroxisome re-assembly also occurs in mutant strains, which are known to lack any peroxisomal membrane remnants, upon re-introduction of the defective gene. Examples of this include *P. pastoris pex3* [98] and *S. cerevisiae pex19* [103] null mutants, *H. polymorpha pex3* [100] and *ts6* [165] mutants, as well as human *pex16* mutants [101]. This indicates that alternative modes of peroxisome assembly may exist. It has been suggested that new peroxisomes may arise from the endomembrane system, e.g. from specialized regions of the ER. South and Gould [101] presented an interesting model for peroxisome re-assembly in human *pex16* mutant cells and suggested that they may arise by conversion from an unknown pre-peroxisomal structure, mediated by Pex16p. It remains unclear whether this model reflects the WT situation or that it serves as rescue machinery to peroxisome formation. However, as in *S. cerevisiae* a *PEX16* homologue has not been identified yet, the human model may not be generally valid. Additional research is therefore required for elucidating this problem in both human and yeast.

Characteristic for various *pex5* mutants (e.g. of *P. pastoris* [151], *S. cerevisiae* [37], *Y. lipolytica* [16, 17, 31]) is the cytosolic location of PTS1 proteins together with the presence of peroxisomal membrane remnants or, in case PTS2 proteins are synthesized (e.g. during growth of yeast on oleate), small peroxisomes. A similar phenotype is described for *H. polymorpha pex5* cells, grown under conditions that both PTS1 and PTS2 proteins are induced [33]. Conversely, in *H. polymorpha pex5* cells, grown on glucose or ethanol in the presence of ammonium sulphate as sole nitrogen source (conditions that fully repress synthesis of the PTS2 protein AMO), peroxisomal membrane remnants were below the limit of detection. On the other hand, peroxisomal remnants were detectable in *H. polymorpha pex5*, when grown at high peroxisome induction conditions on methanol. Therefore, the proliferation of these structures is dependent on the growth conditions and most likely related to the ultimate level of essential peroxisomal membrane proteins.

The membrane remnants in *H. polymorpha pex5* cells display several characteristics, typical for normal WT peroxisomes. They are the target for artificially produced Pex10p.myc, proliferate under specific growth conditions (this study) and are susceptible to selective degradation [96, 109].

Our data demonstrated that newly produced AMO protein accumulated at the Pex10p.myc-containing, pre-existing membrane structures, originally present in the methanol/ammonium sulphate-grown cells. Since the new peroxisomes developed under conditions in which the synthesis of Pex10p.myc was fully repressed, the co-localization of Pex10p and AMO suggests that these organelles originate from Pex10p.myc containing structures. Whether the development of the peroxisomal membrane remnants involved fusion with small pre-peroxisomal AMO-containing vesicles, as for instance demonstrated for matrix protein import in *Y. lipolytica*, is yet unknown [83].

The possibility that the failure of a portion of the peroxisomal structures to import AMO is due to the presence of Pex10p.myc, is not very likely. We showed that Pex10p.myc is functional and can complement the *pex10* mutant phenotype.

In conclusion: our data suggest that peroxisomal membrane remnant structures present in glycerol/methanol/ammonium sulphate-grown *H. polymorpha pex5* cells can develop into peroxisomes upon subsequent synthesis of the PTS2 protein AMO. Notably, this also happens when the cells are placed in conditions (shift from glycerol/methanol/ammonium sulphate to glucose/methylamine) that induce two oppositely directed processes, namely...
degradation of the Pex10p.myc containing peroxisomal structures versus the need for a protein import-competent structure. Essentially similar results have been obtained on *H. polymorpha* WT cells, shifted from methanol/methylamine to glucose/methylamine [166]. Given the rate of Pex10p.myc degradation, our results lend support to the view that few, import-competent structures escape degradation and remain available for peroxisome formation. Apparently, these structures maintained the functions essential for PTS2 protein import of matrix proteins and thus could develop into AMO-containing peroxisomes. These results are in line with recent data on a *PEX5*-deficient CHO cell line by Yamasaki et al. [167]. These authors showed that peroxisomes reappeared after microinjection of Pex5p together with GFP fused to a PTS1 signal. They observed GFP fluorescence in particulate structures in which PMP70 colocalized, suggesting that all peroxisomal remnants were able to gradually take up PTS1 proteins in time. The reasons for the apparent discrepancy with the structures in *H. polymorpha pex5* cells are unknown. A possible explanation may be related to the fact that microinjection in fact confronts the cell with an excess of matrix proteins. In view of the finding that under WT conditions the maximal import capacity of peroxisomes apparently is not fully used [168] it can be envisaged that the sudden excessive amounts of matrix proteins disturbs the normal balance between matrix protein synthesis and import capacity thus leading to additional protein import in existing organelles. However, more research is required to solve this specific question.

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