Sorting of matrix proteins to peroxisomes in the methylotrophic yeast Hansenula polymorpha

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Perspectives

Our current knowledge on the principles of peroxisome homeostasis (biogenesis versus selective degradation) is strongly founded on genetic and ultrastructural approaches and has advanced to a level that several of the essential players of these processes are now known. Nevertheless, the know-how on the principles of these processes is still very limited. Likely, some of the key players are still unidentified. Therefore, additional techniques may be required to deepen our insights e.g. to understand the in vivo sequence and nature of protein-protein interactions of the matrix protein import cascade and the initial events in organelle biogenesis, multiplication and turnover.

Matrix proteins are synthesized in the cytosol and thus have to pass the peroxisomal membrane to reach their final destination. The mechanisms of this translocation process are not yet known and several models have been proposed to reconcile all experimental data. One of these hypotheses that matrix proteins are translocated via a proteinaceous translocation machinery in a comparable mode as for instance the Sec system in prokaryotes and mitochondrial protein import. This model would involve:

a) binding of the matrix proteins by the PTS receptor in the cytosol. For PTS1 proteins this binding process is well understood and thought to initiate the actual import process.

b) docking of the PTS1 receptor/cargo complex at the peroxisomal membrane. Two protein complexes, containing at least Pex13p, Pex14p, Pex17p and a second one, containing Pex10p and Pex12p, are believed to function in this process [150]. It can be envisaged that both complexes reflect an import cascade with different affinities for the receptor/cargo complex.

c) translocation and dissociation of the PTS1 receptor/cargo complex. The above interactions could lead to the dissociation of the cargo from the receptor and the handover of the cargo molecule to the translocation machinery. In *H. polymorpha* evidence was obtained that the Pex5p/cargo complex may be completely translocated across the peroxisomal membrane, followed by dissociation of the complex in the peroxisomal membrane [36].

d) receptor recycling. It is now widely accepted that the PTS1 receptor cycles between the cytosol and the peroxisomes. Hence, import of the receptor/cargo complex into the matrix would imply that a Pex5p export machinery exists. Indeed, in *H. polymorpha* we have obtained evidence for this pathway, in which Pex4p, an ubiquitin-conjugating enzyme, plays a role [35].

Some findings argue against this model. These include for instance that large folded macromolecules (including gold particles up to 9 nm) can pass the peroxisomal membrane [72]. This would require a pore that should easily be detectable by ultrastructural methods but on the other hand has not been identified yet. Alternative models therefore include endocytosis-like events [190] or, more recently, vesicle trafficking and fusion processes [83]. However, how the proteins would enter these putative vesicles is completely unknown.

Obviously, one of the main challenges in peroxisome research is to understand matrix protein import at the molecular level. As genomics have not led to distinct clues yet, other approaches are essential. One of these includes the isolation and analysis of conditional mutants. These may help to identify putative novel proteins that have not been found by the conventional methods because they are essential for the
viability of the cells. Also proteomics may become an important tool in the very near future. With the development of advanced biochemical methods (e.g. blue native gel electrophoresis and analytical 2D gel-electrophoresis) we may be able to identify novel proteins, which are not readily found by genetic methods because of gene redundancy or in case of very small proteins. The rapid advance in genome sequencing will greatly facilitate to clone the genes encoding such proteins.

Another approach to analyze the different steps of matrix protein import is to ‘arrest’ the Pex5p/cargo complex in a specific stadium in the import machinery. This may be accomplished by deleting genes encoding essential components, or by introducing specific mutations in such genes. The subcellular localization of the PTS1 receptor in such strains gives a clue in which step matrix protein import is blocked. Initial experiments have proven the strength of this method e.g. in case of *H. polymorpha pex4* [35] and *pex14* [34], *S. cerevisiae pex8* [51], human *pex10* and *pex12* [49].

Also, non-invasive techniques will be of major importance in the near future to unravel the sequence and nature of protein-protein interactions in the matrix protein import cascade. In particular confocal laser scanning microscopy techniques are suitable for this purpose, e.g. Fluorescence Resonance Energy Transfer (FRET) analysis of different green fluorescent protein (GFP) fusion proteins or Fluorescence Correlation Microscopy (FCM) [191, 192]. For instance, transient protein-protein interactions during the docking of PTS receptor/cargo complex or the translocation across the membrane can be studied by this method. In addition, CLSM can monitor the dynamics of peroxisomal membrane transport or protein import. These techniques will link up with *in vitro* biophysical techniques to analyse protein-protein interactions and will undoubtedly lead to further insights in the complex and dynamic process of peroxisome homeostasis in the near future.

Finally, studies have to be initiated to understand the function of the organelles. This area of peroxisome research is almost a complete black box but of utmost importance to tackle in view of the important implications (e.g. in understanding human peroxisomal disorders and the production of β-lactam antibiotics by certain fungi). The proposed function of the organelles predicts that various transport systems have to exist in the peroxisomal membrane. However, none of them is characterized yet. Another puzzling aspect in this respect is the finding that in freeze etch replicas the fracture faces of the peroxisomal membrane are invariably largely smooth, suggesting that large integral membranes are absent. Also, the protein-phospholipid ratio of peroxisomes is remarkably low, compared to other organelles. Much has therefore to be done to shed light on these virtual contradictions and resolve how metabolites pass the peroxisomal membrane.
Summary

Cell organelles are characteristic for eukaryotic cells. Each organelle fulfills a specific set of tasks that are essential for proper functioning and multiplication of the cells. For instance, the nucleus is a compartment where the genetic information (DNA) is stored, the mitochondrion serves as a 'powerhouse' for the cell, whereas the vacuole/lysosome is the site of waste recycling. A complex network of vesicular structures (the endoplasmic reticulum and Golgi apparatus) activate and modify proteins to be secreted and perform a conscientious quality control before proteins leave the cell. The function of microbodies (peroxisomes/glyoxysomes/glycosomes) is less easy to define. Microbodies consist of a single membrane that encompasses a proteinaceous space, the matrix. The microbody matrix can be characterized as a concentrated solution of enzymes, which determines the physiological role of the organelle. The enzymic content can be highly diverse and dependent on the organism, cell type, or - for yeasts and filamentous fungi - growth conditions. Typical microbody enzymes are enzymes involved in β-oxidation of fatty acids, hydrogen peroxide producing oxidases, catalase and enzymes of the glyoxylate cycle. However, microbodies of totally different function also exist. An example is the glycosome which contains the full set of glycolytic enzymes. This organelle is found in trypanosomes, parasites that cause sleeping sickness. In man microbodies are most abundant in liver cells, where they play a key role in the oxidation of very long chain fatty acids (VLCFAs). When microbodies are absent or malfunctioning in man, due to an inherited peroxisomal disorder, VLCFAs accumulate in the bodies of these patients, causing severe abnormalities that result in an early death. In plant the organelles are important for the mobilisation of storage fat during seed germination and in green leaves for photorespiration. In yeasts and filamentous fungi microbodies play crucial roles in the primary metabolism of various carbon and nitrogen sources (e.g. alkanes, fatty acids, methanol, primary amines). Few biosynthetic enzymes have been found in microbodies. In man microbody enzymes are involved in ether-lipid and cholesterol biosynthesis. Another example is penicillin biosynthesis in Penicillium chrysogenum.

Despite the diversity in microbody function, the mechanisms involved in the formation of the organelle (microbody biogenesis) are very similar among eukaryotes indicating that these have been strongly conserved during evolution. Hence, knowledge obtained from research on simple model organisms like yeasts are useful for to higher eukaryotes, including man.

The studies described in this thesis focus on the molecular mechanisms involved in peroxisome biogenesis in the methylotrophic yeast Hansenula polymorpha. In this yeast peroxisomes massively develop during growth on methanol. Under these growth conditions three key enzymes of methanol metabolism are found in peroxisomes, namely alcohol oxidase (AO), dihydroxyacetone synthase (DHAS) and catalase (CAT). Depending on the growth substrates other sets of enzymes can be found in H. polymorpha peroxisomes. For instance, when cells are grown in the presence of primary amines as a sole source of nitrogen the peroxisomes harbor amine oxidase and catalase. In glucose/ammonium sulfate grown cells, only a few, small microbodies are present, because the metabolism of these growth substrates does not involve peroxisomal enzymes. Upon transfer of glucose-grown cells to methanol-containing media the number and size of the organelles rapidly increase. The generally accepted view is that newly
Summary

Synthesized peroxisomal matrix proteins are imported into the few, small organelles present in the inoculum cells. The organelar membrane grows as a result of the incorporation of membrane proteins and lipids. When the organelle has reached its mature size a new organelle buds off from the pre-existing one. In recent years quite some progress has been made in elucidating the molecular mechanisms of peroxisome biogenesis (matrix protein import, insertion of membrane proteins, organelle division). At present 14 *H. polymorpha* PEX genes, involved in peroxisome biogenesis, have been cloned and sequenced. Recently, also the first *H. polymorpha* genes involved in selective peroxisome degradation (PDD) have been described. An overview of the recent achievements is presented in chapter 1.

Several indirect lines of evidence indicated that the endoplasmic reticulum (ER) plays a role in the formation of the peroxisomal membrane. A hypothetical model based on these findings predicts that certain peroxisomal membrane proteins first insert into the ER membrane. Subsequently, vesicles are formed from the ER that contain these membrane proteins and also lipids. Finally, fusion of these vesicles with pre-existing peroxisomes results in the incorporation of the vesicle components into the peroxisomal membrane.

To test this hypothesis in *H. polymorpha* the fungal toxin brefeldin A (BFA) was used (Chapter 2). BFA is known to inhibit the formation of vesicles at the endoplasmic reticulum (ER). Electron microscopical studies revealed that, as expected, the addition of BFA to cultures of *H. polymorpha* wild type cells caused morphological changes to the ER. However, in methanol-grown cells it was evident that also the development of peroxisomes was partially hindered. Immunocytochemical experiments revealed that both peroxisomal membrane proteins (Pex3p and Pex14p) and matrix proteins (AO, DHAS, CAT and Pex8p) accumulated at the ER and were transferred to peroxisomes upon removal of BFA. Most likely, specific peroxisomal membrane proteins, which are essential for organelle biogenesis, are sorted to peroxisomes via the ER and ER-derived vesicles. In the presence of BFA the formation of these vesicles is inhibited resulting in the accumulation of peroxisomal membrane proteins at the ER. Accumulation of membrane proteins involved in matrix protein import (e.g. Pex14p) at the ER may explain why also peroxisomal matrix proteins were mistargeted.

Several genes essential for peroxisome biogenesis (*PEX* genes) are involved in the import of matrix proteins. Pex5p is the receptor of the carboxy terminal Peroxisomal Targeting Signal 1 (PTS1), whereas Pex7p recognizes the N-terminal PTS2 of matrix proteins. Upon synthesis in the cytosol, the PTS of the matrix protein is recognized by the corresponding receptor, Pex5p or Pex7p. Subsequently, the receptor/cargo complex is thought to bind to a proteinaceous docking site on the peroxisomal membrane, upon which the matrix protein is translocated across the peroxisomal membrane. Whether the receptors are co-imported with the cargo proteins is still a matter of debate. Finally, the receptors are recycled to the cytosol where they can mediate new rounds of import.

Deletion of the *PEX5* gene (*pex5*) leads to a complete block in PTS1 protein import. Under conditions that induce PTS1 proteins but fully repress the major PTS2 protein AMO, *H. polymorpha* *pex5* cells contain peroxisomal membrane structures that lack matrix proteins, but in which peroxisomal membrane proteins are normally inserted. In chapter 3 evidence is presented that these membrane structures can develop into peroxisomes upon induction of the PTS2 protein AMO. For this purpose peroxisomal membrane structures in *H. polymorpha* Δpex5 cells were tagged with a Pex10p.myc fusion protein. Cells were pre-grown under conditions that induce the Pex10p.myc
containing peroxisomal membranes, but fully repress the synthesis of the PTS2 protein AMO. Subsequently, these cells were shifted to a growth medium that fully repressed the synthesis of Pex10p.myc, but induced AMO. Biochemical and electron microscopical experiments revealed that 2 hours after the shift small peroxisomes were present in the cells that contained both Pex10p.myc and AMO.

Pex14p is thought to be a component of the proteinaceous receptor docking site at the peroxisomal membrane, because domains of this protein that are exposed to the cytosol, can physically interact with Pex5p and Pex7p. Moreover, deletion of the PEX14 gene results in a major defect in both PTS1 and PTS2 protein import. Unexpectedly, overproduction of the PTS1 receptor Pex5p in a H. polymorpha Δpex14 deletion strain largely restored the import defect of the major PTS1 proteins AO and DHAS but not of the PTS2 protein AMO (chapter 4). This finding indicates that PTS1 matrix protein import can function, although at reduced efficiency, in the absence of Pex14p. Biochemical analysis of purified peroxisomal fractions of H. polymorpha Δpex14 overproducing Pex5p revealed that large amounts of Pex5p accumulated at the outer surface of the peroxisome. Hence, association of Pex5p to the peroxisomal membrane does not necessarily involve the presumed docking site component Pex14p.

Several studies (two-hybrid studies and co-immuneprecipitations) have suggested the presence of large protein complexes in peroxisomal membranes. An example is the putative receptor docking complex that is thought to contain Pex13p, Pex14p and Pex17p. Other presumed protein complexes are involved in membrane protein insertion or solute transport. Although the presence of these protein complexes is generally accepted, they have never been demonstrated directly. Using blue native gel electrophoresis, several relatively stable protein complexes of different sizes were shown to be present in the peroxisomal membrane of methanol-grown H. polymorpha wild type cells (chapter 5). Pex14p was found in a protein complex of approximately 400-500 kDa together with a number of other proteins.