Summary

Microbodies constitute a relatively recently (1954) discovered class of subcellular organelles which are present in all eukaryotic cells. Depending on their metabolic function, microbodies have been subdivided in peroxisomes, glyoxysomes, glycosomes and hydrogenosomes. Microbodies are composed of a homogeneous, sometimes crystalline, matrix which is surrounded by a single membrane. The size and number of organelles per cell is generally influenced by the environmental conditions. For instance, when yeast cells are grown under conditions which require the presence of one or more peroxisomal matrix enzymes, the volume fraction of peroxisomes in the cell may increase from <0.1 (growth on glucose) to >30% (growth on methanol). This demonstrates that peroxisomes are inducible in nature. The metabolic importance of peroxisomes in man is examplified by the fact that absence of these organelles may lead to severe abnormalities, sometimes followed by an early death (*e.g.* Zellweger syndrome). Consequently, many studies have been devoted to unravel the molecular mechanisms of peroxisome biogenesis and function. Yeasts are excellent model systems for such studies in view of the following features: (*i*) the induction and protein composition of peroxisomes can readily be manipulated by varying growth conditions, (*ii*) in the absence of peroxisomes, yeasts are viable and (*iii*) they are readily accessible for classical and molecular genetics, biochemistry, detailed studies of physiology and morphology. Since the late 1980's, many peroxisome-deficient mutants have been isolated from a variety of yeast species and the corresponding genes have been cloned and characterized. In recent years, these yeast sequences have been succesfully used to identify homologuos genes (13 out of 21 known to date) of mammals by screening the ever increasing sequence data available in the current databases, demonstrating the value of using yeast, in studies of peroxisome biogenesis and function.

The initial morphological studies of the 1960's and 1970's lend support to the notion that new peroxisomes were formed as buds from the endoplasmic reticulum. Subsequently, biochemical analysis demonstrated that peroxisomal matrix and membrane proteins are synthesized in the cytosol and are post-translationally imported into the peroxisomes. Together with morphological studies, which showed that upon import of proteins the microbodies grow in size until they maturate and bud off a new organelle, this led to the model that peroxisomes arise by 'growth and division' from pre-existing ones.

Over 20 *PEX* genes, which are essential for peroxisome biogenesis and function, have now been identified and characterized in various organisms (see Table 1). Most of their protein products called peroxins are peroxisomal membrane proteins. Recent advances in our understanding of the structure-function relationships of these and other peroxisomal membrane proteins is reviewed in chapter 1.

In this thesis the methylotrophic yeast *Hansenula polymorpha* was used as a model organism to study peroxisome biogenesis. *H. polymorpha* cells are able to grow on methanol as a sole carbon source, which requires the compartmentalization of part of its metabolism in peroxisomes. The specific aim of this study was to investigate the molecular mechanisms underlying the biogenesis of the peroxisomal membrane. In the early 1990's *H. polymorpha* mutants have been isolated and selected for defects in peroxisome function, primarily by their inability to grow on methanol. One of these mutants, which lacked any detectable peroxisomal membrane structures, was studied in detail.

Chapter 2 describes the cloning and initial characterization of the *H. polymorpha PEX3* gene. This gene encodes an integral peroxisomal membrane protein of 52-kDa. The absence of Pex3p has a dramatic affect on peroxisome biogenesis. Peroxisomes and even peroxisomal membrane remnants were completely undetectable under methanol inducing conditions in *pex3* defective cells, suggesting that Pex3p is involved in the early stages of peroxisome biogenesis. The reintroduction of *PEX3* in cells lacking this gene results in a rapid reappearance of peroxisomes, indicating that the organelles do not necessarily derive from pre-existing peroxisomes. When, after re-establishing peroxisome biogenesis, *PEX3* expression was switched of, peroxisomes were observed to disintegrate over time in a manner that suggested that Pex3p may be required for maintenance of the integrity of the peroxisomal membrane. Studies to identify sequences in Pex3p involved in sorting of the protein to the peroxisomal membrane demonstrated that these were located in the amino-terminus of the protein. Subsequent experiments demonstrated that short N-terminal fragments of Pex3p were capable of targeting a reporter protein to the ER, suggesting that the sorting of Pex3p to the peroxisomal membrane may proceed via the ER.

In order to elucidate the role of Pex3p in the biogenesis and function of peroxisomes conditional *PEX3* mutants were constructed in which the rate of synthesis of Pex3p can be regulated. The relation between Pex3p levels in the cell and peroxisome formation was studied in wild type and *pex3* deletion strains (chapter 3 and 4). In chapter 3, data is presented indicating that peroxisome biogenesis can be manipulated by varying the levels of Pex3p. The results demonstrate that in wild type cells the expression of the *PEX3* gene is carefully regulated in order to ensure proper peroxisome assembly and function in *H. polymorpha*. High steady-state levels of Pex3p strongly interfere with peroxisomal matrix import and induced the formation of numerous small vesicles. In contrast, an initial sharp increase in Pex3p levels in a relative short time interval induced excessive proliferation of small peroxisomes (chapter 4). These organelles harboured characteristic features of glucose-peroxisomes, since they were all import-competent for matrix proteins and were not susceptible to glucose-induced

degradation, unlike mature organelles. These results suggest that peroxisome proliferation and matrix protein import are coupled processes in *H. polymorpha* which are performed by protein complexes in the peroxisomal membrane. The protein complexes are thought to be functional as long as the stoichiometry of its components is not significantly changed, for instance by overproducing one of the components.

Chapter 5 describes a detailed mutational analysis in order to define the targeting sequence in the N-terminus of *H. polymorpha* Pex3p. In the N-termini of all Pex3p-orthologues a highly conserved sequence of positively charged amino acids is present which was the target of these studies. Substitution of all positively charged amino acids in the conserved sequence to uncharged residues led to a mutant Pex3p which was only partially able to restore peroxisome assembly and function in *Δpex3* cells. Unexpectedly, the level of the mutant protein in these cells was significantly decreased compared to the levels of the wild type protein. This decrease could reflect an instabile mutant protein which may be caused by it's mistargeting. When proteasome function was inhibited by the addition of an inhibitor, increased levels of instabile mutant Pex3p were obtained, indicating that these proteins are actively degraded by the proteasome. Although the positively charged amino acids are highly conserved in all Pex3p's, substitutions of single or two positively charged amino acids to uncharged or even negatively charged residues, did not effect the functionality of Pex3p. Mutating more than two positively charged amino acids affected the targeting of Pex3p and ultimately resulted in decreased levels of mistargeted mutant protein when additional mutations were introduced. The decrease in functional Pex3p was shown to negatively affect the levels of other membrane-bound peroxins, such as Pex10p and Pex14p. These results suggest that functional Pex3p is necessary in order to synthesize and/or maintain stable protein complexes containing these peroxins.

Table 1. List of peroxins, proteins essential for peroxisome biogenesis

Peroxin (Mw)[1]	Human Homolog (PBD CG)[2]	Characteristics[3]	Interacts with	References
Pex1p (117-143)	+ (1)	AAA-type ATPase; contains two AAA domains	Pex6p	1-8
Pex2p (31-52)	+ (10)	IPMP; C-terminal Zn-finger motif; N-glycosylated; sorted via ER[4]		9-17
Pex3p (42-52)	+	IPMP; *Δpex3* cells do not contain ghosts	Pex19p	18-21
Pex4p (21-24)	-	PMP; ubiquitin-conjugating enzyme		22-24
Pex5p (64-69)	+ (2)	PTS1-receptor; 6-7 TPR domains	Pex13p, Pex14p	25-30
Pex6p (112-127)	+ (4)	AAA-type ATPase; contains two AAA domains	Pex1p	6-8, 31-36
Pex7p (37-42)	+ (11)	PTS2-receptor; 7 WD40 domains	Pex13p, Pex14p	37-42
Pex8p (71-81)	-	PMP facing matrix; contains PTS1 and PTS2		43-45
Pex9p (45)	-	IPMP		46
Pex10p (34-48)	+ (7/B)	IPMP; C-terminal Zn-finger motif		47-50
Pex11p (24-31)	+	IPMP; in mammals 2 isoforms (α/β); α-form contains dilysine motif, binds coatomer/ARF	Pex11p	51-60
Pex12p (40-46)	+ (3)	IPMP; C-terminal degenerate Zn-finger motif		61-63
Pex13p (40-43)	+	IPMP; cytosolic SH3 domain	Pex5p, Pex7p, Pex14p	64-66
Pex14p (38-41)	+	PMP; facing cytosol; phosphorylated	Pex5p, Pex7p, Pex13p, Pex17p	67-71
Pex15p (44)	-	IPMP; phosphorylated; O-mannosylated		72
Pex16p (39-44)	+ (9/D)	PMP; facing matrix; N-glycosylated; sorted via ER[4]		73-75
Pex17p (23)	-	PMP; facing cytosol	Pex14p	76
Pex18p (32)	-	involved in PTS2-targeting; primarily cytosolic, partially bound to the peroxisomal membrane (faces the cytosol)	Pex7p, Pex21p	77
Pex19p (33-40)	+	PMP; facing cytosol; farnesylated	Pex3p	78-80
Pex20p (47)	-	involved in oligomerization and targeting of thiolase		81
Pex21p (33)	-	involved in PTS2-targeting; cytosolic	Pex7p, Pex18p	77

1. Mw - Molecular weight in kDa.
2. PBD CG - Complementation Groups (numbering, see Shimozawa *et al.* (1998) Am. J. Hum. Genet. 63, 1898-1903) of fibroblast cell lines obtained from patients with Peroxisomal Biogenesis Disorders.
3. (I)PMP - (Integral) Peroxisomal Membrane Protein.
4. in the yeast *Yarrowia lipolytica*.

References, see page 110.

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