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Peroxisome biogenesis in *Saccharomyces cerevisiae*

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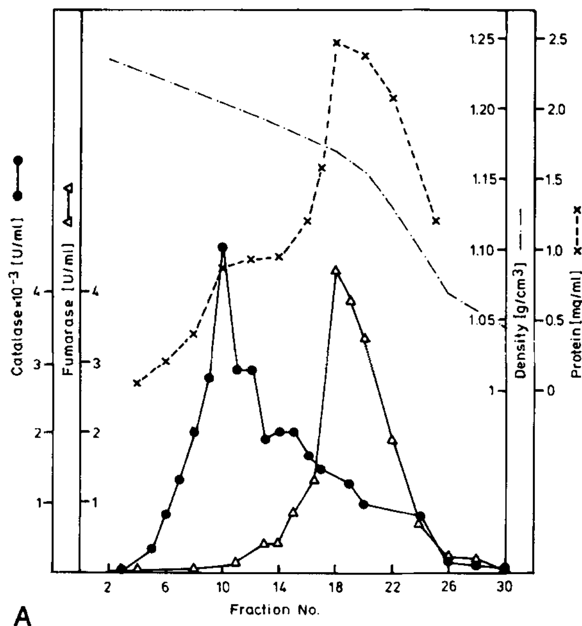
Abstract

The observation that peroxisomes of *Saccharomyces cerevisiae* can be induced by oleic acid has opened the possibility to investigate the biogenesis of these organelles in a biochemically and genetically well characterized organism. Only few enzymes have been identified as peroxisomal proteins in *Saccharomyces cerevisiae* so far; the three enzymes involved in β-oxidation of fatty acids, enzymes of the glyoxylate cycle, catalase A and the PAS3 gene product have been unequivocally assigned to the peroxisomal compartment. However, more proteins are expected to be constituents of the peroxisomes in *Saccharomyces cerevisiae.*

Mutagenesis of *Saccharomyces cerevisiae* cells gave rise to mutants unable to use oleic acid as sole carbon source. These mutants could be divided in two groups: those with defects in structural genes of β-oxidation enzymes (fox-mutants) and those with defects in peroxisomal assembly (pas-mutants). All fox-mutants possess morphologically normal peroxisomes and can be assigned to one of three complementation groups (FOX1, 2, 3). All three FOX genes have been cloned and characterized. The pas-mutants isolated are distributed among 13 complementation groups and represent 3 different classes: peroxisomes are either morphologically not detectable (type I) or present but non-proliferating (type II). Mislocalization concerns all peroxisomal proteins in cells of these two classes. The third class of mutants contains peroxisomes normal in size and number, however, distinct peroxisomal matrix proteins are mislocalized (type III). Five additional complementation groups were found in the laboratory of H.F. Tabak. Not all PAS genes have been cloned and characterized so far, and only for few of them the function could be deduced from sequence comparisons.

Proliferation of microbodies is repressed by glucose, derepressed by non-fermentable carbon sources and fully induced by oleic acid. The regulation of four genes encoding peroxisomal proteins (PAS1, CTA1, FOX2, FOX3) occurs on the transcriptional level and reflects the morphological observations: repression by glucose and induction by oleic acid. Moreover, trans-acting factors like ADR1, SNF1 and SNF4, all involved in derepression of various cellular processes, have been demonstrated to affect transcriptional regulation of genes encoding peroxisomal proteins.

The peroxisomal import machinery seems to be conserved between different organisms as indicated by import of heterologous proteins into microbodies of different host cells. In addition, many peroxisomal proteins contain C-terminal targeting signals. However, more than one import route into peroxisomes does exist. Dissection of the import mechanism in a genetically well suited organism like *Saccharomyces cerevisiae* together with further characterization and functional assignment of the PAS gene products will provide insight into the biogenesis of peroxisomes. Moreover, these studies will lead to a good model system for elucidation of the mechanisms underlying human peroxisomal disorders.



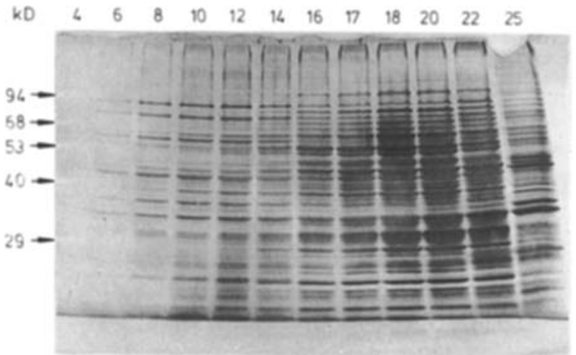


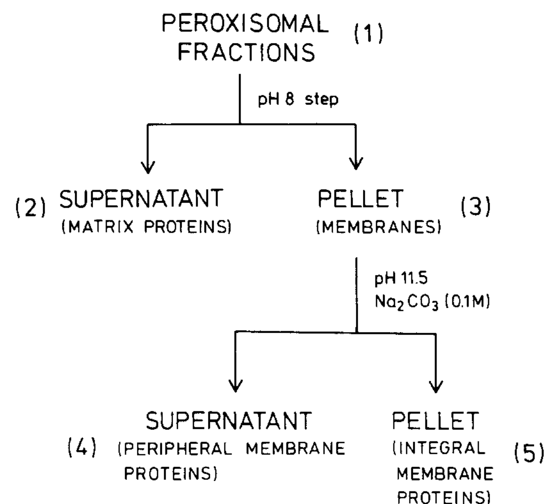
Fig. 1. Subcellular fractionation of an organellar pellet (25.000 × g) from oleic acid-grown cells. (A) Sucrose density gradient; (B) SDS-PAGE analysis of selected gradient fractions. 8 μl of each fraction were applied to each lane.
peroxisomal peak: fraction 10.
mitochondrial peak: fraction 18.

Introduction

The budding yeast *Saccharomyces cerevisiae* is well suited for investigating fundamental questions of cell biology because of the ease with which it can be manipulated and because of the wealth of information available concerning its physiology, biochemistry, and genetics (Rose & Harrison 1987–1991). In addition, in recent years recombinant DNA procedures have opened in an unprecedented way new avenues to analyze and manipulate the yeast genome (Botstein & Fink 1988). Combined genetic, biochemical and molecular genetic approaches have been used very successfully to investigate the biogenesis of subcellular structures, including mitochondria (Glick et al. 1991; Pfanner et al. 1991), endoplasmic reticulum (Newman & Ferro-Novick 1990; Hicke & Schekman 1990), vacuoles (Klionsky et al. 1990; Rothman et al. 1989), and nuclei (Silver 1991). Peroxisomes are late comers to this field.

The history of peroxisomes in yeasts began when Avers and Federman demonstrated the presence of these organelles in *S. cerevisiae* (Avers & Federman 1968). However, in contrast to other fungal species (Tanaka & Fukui 1989; Veenhuis & Harder 1991) thereafter very little was heard of peroxisomes in *S. cerevisiae*. Their presence in *S. cerevisiae* was even questioned when the particulate catalase (catalase A) was assigned to vacuoles (Susani et al. 1976). These initial observations can be explained in view of our current knowledge because peroxisomes are rare and small under conditions originally chosen for growth and isolation (Veenhuis et al. 1987).

Accumulated evidence has demonstrated that proliferation of peroxisomes in yeasts is under the control of glucose repression and derepression as well as induction by distinct carbon and nitrogen sources (Harder & Veenhuis 1989; Tanaka & Fukui 1989; Veenhuis & Harder 1991). This inducibility of peroxisome proliferation in yeasts and also to some extent in higher eukaryotes (animals and plants) reflects one specific feature of these organelles. Another striking property is their functional diversity. Their enzyme patterns vary markedly depending on organism, cell type, and environmental conditions (Fahimi & Sies 1987). Thus, media differing in carbon and nitrogen sources lead to induction of different peroxisomal metabolic pathways in cells of various species of yeasts and filamentous fungi. Especially thoroughly studied examples are *Hansenula polymorpha, Pichia pastoris* (Gleeson & Sudberry 1988), and *Neurospora crassa* (Wanner & Theimer 1982, Desel et al. 1982, Kunau et al. 1987). In contrast, for many years no



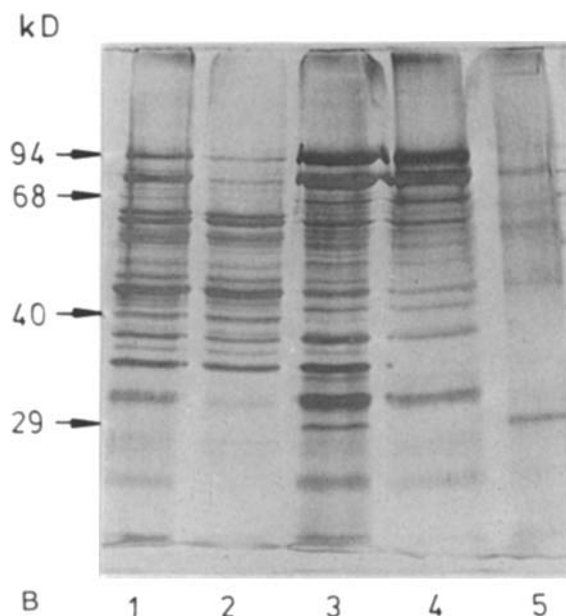


Fig. 2. (A) Subfractionation scheme of gradient fractions containing peroxisomes; (B) SDS-PAGE analysis of peroxisomal subfractions. Numbers correspond to numbers given in brackets in Fig. to A: (1) isolated peroxisomes (sucrose density gradient); (2) pH-8-step: supernatant (matrix proteins); (3) pH-8-step: pellet (membrane proteins); (4) pH-11.5-step: supernatant (peripheral membrane proteins); (5) pH-11.5-step: pellet (integral membrane proteins) about 15 μg of proteins were applied to each lane.

progress was made regarding biogenesis of peroxisomes in *S. cerevisiae* despite many efforts of various laboratories. In a very fruitful collaboration between the laboratory of one of us (Wolf-H. Kunau) and that of Marten Veenhuis (University of Groningen/The Netherlands) the carbon source oleic acid was identified as inducer of both the peroxisomal β-oxidation and the proliferation of peroxisomes in *S. cerevisiae* (Veenhuis et al. 1987). This observation started the renaissance of peroxisomal research in this yeast. Since oleic acid is still the only peroxisomal inducer known for *S. cerevisiae*, in this respect this organism lags behind other yeasts. Nevertheless, the extensive knowledge of its biochemistry, genetics and molecular biology together with the possibility to induce its peroxisomes makes *S. cerevisiae* a well suited organism to study peroxisome biogenesis.

The purpose of this article is to review the first attempts to dissect the mechanism of peroxisome biogenesis by a combined biochemical and genetic approach. To do this we will describe (i) proteins known or suggested to be constituents of peroxisomes in *S. cerevisiae*, (ii) the peroxisomal mutants (fox- and pas-mutants) recently isolated and the first PAS genes which have been characterized, (iii) what is known about the import of peroxisomal proteins and (iv) the regulation of genes encoding peroxisomal proteins. Although focussing on *S. cerevisiae* some results obtained with other yeasts will be included for comparison. However, we make no attempt for an exhaustive coverage of peroxisomal research in other yeasts.

Peroxisomal proteins

Compartmentation of metabolic pathways is essential for eukaryotic cells. Each subcellular structure has its own distinct set of proteins and these proteins have to be imported into their target organelle. The knowledge which proteins are constituents of the organelle under study is a prerequisite for an experimental approach towards organelle biogenesis. For the sake of simplicity it is convenient to classify peroxisomal proteins into mem-

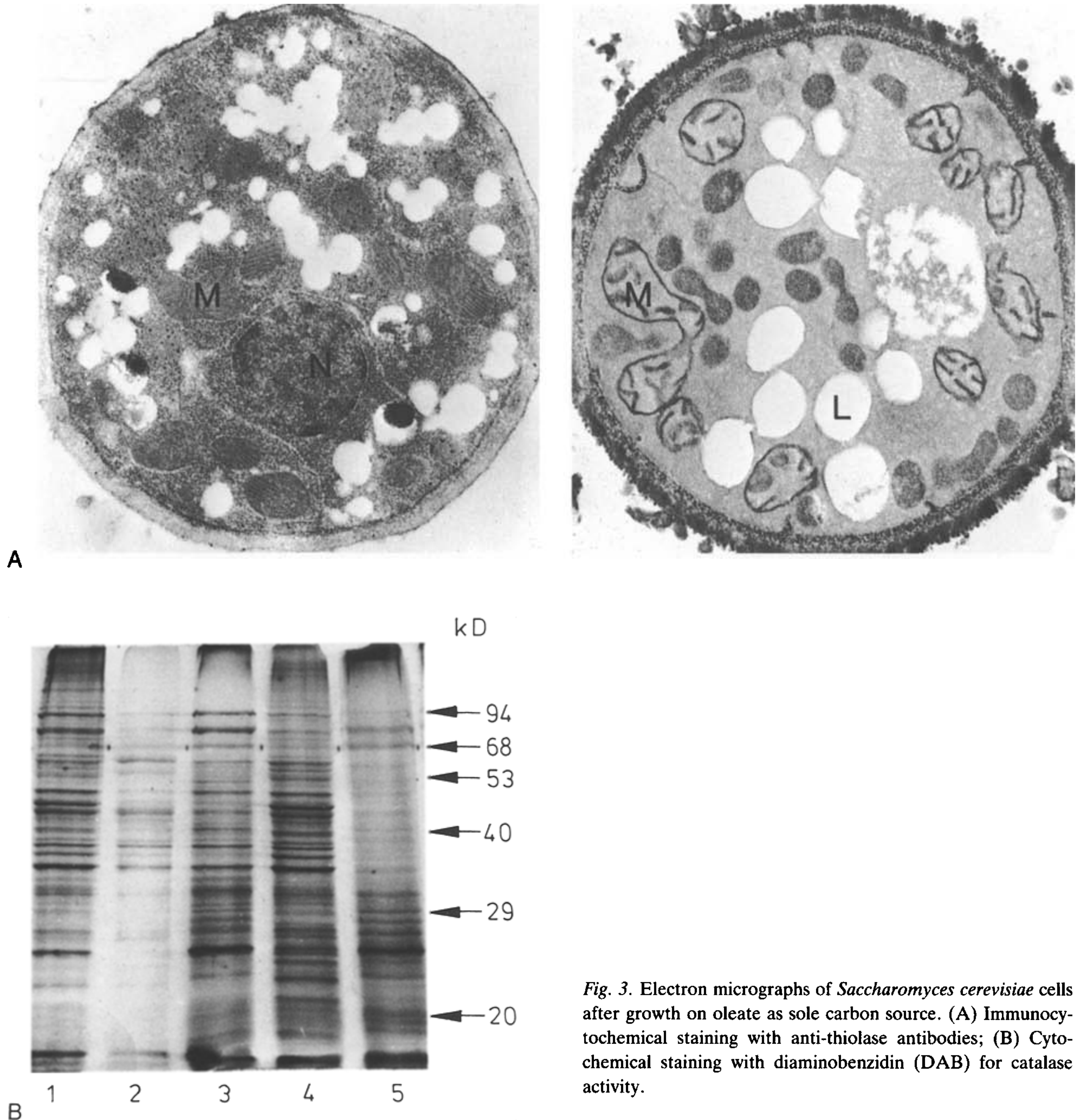


Fig. 3. Electron micrographs of *Saccharomyces cerevisiae* cells after growth on oleate as sole carbon source. (A) Immunocytochemical staining with anti-thiolase antibodies; (B) Cytochemical staining with diaminobenzidin (DAB) for catalase activity.

brane and matrix proteins. In addition, the former ones are frequently subdivided into integral and peripheral membrane proteins based entirely on an operational criterion (solubility in 0.1 M Na-carbonate pH 11,5, Fujiki et al. 1982). Analyses of this kind have been performed for mammalian tissues, especially liver (Lazarow 1984; Hashimoto et al. 1986; Hardeman et al. 1990) and some yeasts, such as *Candida boidinii* (Goodman et al. 1986, 1990) and *H. polymorpha* (Sulter et al. 1990). For *S. cerevisiae* much less is known (McCammon et al. 1990a, see Figs 1 and 2) reflecting the fact that peroxisomes of this yeast only recently became accessible for investigation.

Catalase A was the first and for some time the only peroxisomal matrix protein which was bio-

chemically characterized. Mutants of catalase A and its cytosolic counterpart catalase T were isolated before the peroxisomal localization was firmly established (Spevak et al. 1983; Cohen et al. 1985). In the meantime, catalase A is besides 3-oxoacyl-CoA thiolase (see below) the most frequently used protein for biochemical or immunocytochemical characterization of peroxisomes in *S. cerevisiae* (see Fig. 3). Catalase A has been cloned and sequenced (Cohen et al. 1985, 1988) and the complex regulation of its expression is being studied (Simon et al. 1991).

With the demonstration of a peroxisomal β-oxidation system in *S. cerevisiae* (Veenhuis et al. 1987, Skoneczny et al. 1988) several new peroxisomal enzymes were identified. Peroxisomal β-oxidation systems consist of acyl-CoA oxidase, multifunctional protein and 3-oxoacyl-CoA thiolase. This was demonstrated in mammals (Hashimoto 1990), plants (Kirsch et al. 1986), various fungi (Kunau et al. 1988) and now also in *S. cerevisiae*. All three proteins have been purified and characterized (Kunau et al., in preparation) as well as cloned and sequenced (Dmochowska et al. 1990; Kunau et al., in preparation). These three proteins together catalyze the four reactions of the classical β-oxidation pathway.

While catalase, acyl-CoA oxidase and thiolase are matrix enzymes the multifunctional protein of *S. cerevisiae* is predominantly found in the peripheral membrane protein fraction. Whether peroxisomes of *S. cerevisiae* like other yeasts (Yamada et al. 1980; Numa 1981) also contain a peroxisomal acyl-CoA synthetase is not known.

A second major pathway frequently found in peroxisomes is the glyoxylate cycle. While plant glyoxysomes contain the enzymes of the entire cycle only the key enzymes, isocitrate lyase and malate synthase, have been demonstrated in peroxisomes of fungi (Kindl & Lazarow 1982). However, evidence for the presence of glyoxylate cycle enzymes in *S. cerevisiae* has been controversial. Early reports of the presence of enzymes of the glyoxylate cycle in peroxisomes (Szabo & Avers 1969) contrast with others assigning these enzymes to the cytosol (Duntze et al. 1969, Parish 1975). More recently, the possibility to induce peroxisome proliferation by growth on oleate prompted reinvestigation of this problem. Activities of malate synthase (McCammon et al. 1990a), citrate synthase (Lewin et al. 1990) and malate dehydrogenase (McCammon et al. 1990a; Minard & McAllister-Henn 1991) were demonstrated to co-migrate with peroxisomes in sucrose density gradients. The presence of the putative peroxisomal targeting signal S-K-L (Gould et al. 1989, see below) at the extreme C-termini of the citrate synthase 2 (Rosenkrantz et al. 1986) and malate synthase (A. Hartig et al., unpubl.) reinforced this conclusion. In addition, for malate synthase immunocytochemical evidence also indicated a peroxisomal location (McCammon et al. 1990a). For isocitrate lyase, however, the intracellular location is entirely enigmatic. While subcellular fractionation studies consistently showed more than 95% of the enzyme activity in the soluble fraction (Duntze et al. 1969; McCammon et al. 1990a) preliminary immunocytochemical evidence indicates a peroxisomal location (McCammon et al. 1990a). Although leakage of major fractions of activities have been reported also for other peroxisomal enzymes of *S. cerevisiae* such as malate dehydrogenase and malate synthase (McCammon et al. 1990a) it is not easy to envisage how an enzyme can be lost completely from peroxisomes even before or during the first sedimentation of these organelles from cell lysates. The difficulty to recover these enzymes predominantly in the peroxisomal fraction might reflect the lack of intactness of isolated peroxisomes much more than currently anticipated. Peroxisomes seem to be extremely fragile under conditions used for isolation. To date, it is not easy to assess whether the isolation of intact peroxisomes is a general problem or more pronounced for distinct organisms. The difficulties to set up a reliable *in vitro*-import system for peroxisomes (see below) seems to support the former possibility.

Very little is known about membrane proteins of peroxisomes from *S. cerevisiae* (McCammon et al. 1990a; V. Hines, pers. commun.; W.-H. Kunau, unpubl.). In some other yeasts membrane analyses have been reported (see below). Three peroxisomal membrane proteins of *C. boidinii* have been cloned and sequenced, PMP20 (Garrard & Good-

man 1989), PMP31 (J.M. Goodman, pers. commun.) and PMP47 (McCammon et al. 1990b). Recently, the first primary structure of a peroxisomal integral membrane protein of *S. cerevisiae* (Pas3p) was reported (Höhfeld et al. 1991). As a protein encoded by a PAS gene this membrane protein is essential for peroxisome biogenesis. It has been partially characterized and shown to possess properties expected for a membrane bound receptor. The availability of Pas3p as membrane marker should now greatly facilitate identification of peroxisomal membranes in cells of *S. cerevisiae*. For higher eukaryotes a first integral membrane protein essential for peroxisome formation has also recently been identified (Tsukamoto et al. 1991).

Based on the number of polypeptide bands resolved by SDS-PAGE of peroxisomal fractions of *S. cerevisiae* (Fig. 1) it seems very likely that in the future more proteins will be identified as peroxisomal constituents of this yeast (see Table 1). On the basis of the assumption that peroxisomal import is conserved between different organisms (Gould et al. 1990), potential peroxisomal proteins of *S. cerevisiae* can be listed. A putative peroxisomal targeting signal (Ser-Lys-Leu at the extreme C-terminus, see below) has been identified for the gene products of PAS6 (A. Skaletz & W.-H. Kunau, unpubl.) and of DAL7 (Yoo & Cooper 1989, see Table 1B). Recently, the heterologous import of human peroxisomal Cu,Zn-superoxide dismutase into peroxisomes of *S. cerevisiae* has been reported (Keller et al. 1991). Since the yeast counterpart of this enzyme has a similar primary sequence (Bermingham-McDonogh et al. 1988), it is conceivable that Cu,Zn-superoxide dismutase of *S. cerevisiae* is a peroxisomal protein as well. Activation of fatty acids is a prerequisite for their degradation, and acyl-CoA synthetases have been demonstrated in peroxisomes of mammals (Alexson et al. 1985; Lageweg et al. 1991) and *Yarrowia lipolytica* (Numa 1981). Genetic and biochemical evidence for an acyl-CoA synthetase in *S. cerevisiae* has been reported (Kamiryo et al. 1976), however, the activity was measured only in glucose-grown cells. It seems likely that a second oleic acid-induced synthetase is present, resembling the situation in *Y. lipolytica*. Another candidate for a peroxisomal protein in *S. cerevisiae* is the nonspecific lipid-transfer protein, demonstrated in peroxisomes of mammals (Mori et al. 1991) and *C. tropicalis* (Szabo et al. 1989, Tan et al. 1990). Assuming that induction by acetate or ethanol is an indication for peroxisomal location (e.g. malate synthase, McCammon et al. 1990a) then the alanine-glyoxy-

Table 1. Proteins detected (A) or expected to be (B) in peroxisomes of *Saccharomyces cerevisiae*.

Proteins			Sequence	Localized	C-term.	Gene
A	catalase A	Sc	(1)	px (2)	-SKF	CTA1
	acylCoA-oxidase	Sc	(3)	px (2)	-INK	POX1
	multifunctional pr.	Sc	(4)	px (4)	-SKL	FOX2
	3-keto-thiolase	Sc	(5)	px (5)	-IKE	FOX3
	citrate synthase 2	Sc	(6)	px (7)	-SKL	CIT2
	malate DH 2	Sc	(8)	px (9)	-ASS	MDH2
	isocitrate lyase	Sc	(10)	? (9)	-VKK	ICL1
	malate synthase	Sc	(11)	px (9)	-SKL	MLS1
	PAS3 gene product	Sc	(12)	px (12)	-FKP	PAS3
B	PAS6 gene product	Sc	(13)	no	-SKL	PAS6
	DAL7 gene product (malate synthase?)	Sc	(14)	no	-SKL	DAL7

Abbreviations used: Cb – *Candida boidinii*; Ct – *Candida tropicalis*; Hp – *Hansenula polymorpha*; Sc – *Saccharomyces cerevisiae*. (1) = Cohen et al. 1988; (2) = Skoneczny et al. 1988; (3) = Dmochowska et al. 1990; (4) = K. Hiltunen et al. manuscript submitted; (5) = W.-H. Kunau et al. unpubl.; (6) Rosenkrantz et al. 1986; (7) = Lewin et al. 1990; (8) = Minard & McAllister-Henn 1991; (9) = McCammon et al. 1990a; (10) = E. Fernandez et al. unpubl., EMBL accession nr X61271; (11) = A. Hartig et al. unpubl.; (12) = Höhfeld et al. 1991; (13) = A. Skaletz & W.-H. Kunau, unpubl.; (14) = Yoo & Cooper 1989.

late amino transferase (Takada & Noguchi 1985) should be found in peroxisomes of *S. cerevisiae*, as was reported for other organisms (Sakuraba et al. 1991). If peroxisomal location in other organisms is a first hint for the same location of the protein under study HMG-CoA reductase can be considered a potential candidate, too. In mammalian cells distinct HMG-CoA reductase proteins are present in peroxisomes as well as in the ER (Rusnak et al. 1990). However, in *S. cerevisiae* the products of both genes identified so far are located in the ER (Sengstag et al. 1990) in glucose-grown cells. It would be interesting to look for still another HMG-CoA reductase in oleic acid-grown cells. The fact that mitochondrial import depends on the action of the heat shock proteins hsp70 and hsp60 inside the organelle (Neupert et al. 1990) suggests the existence of hsps also in peroxisomes. However, this hypothesis implies high similarity of the two import mechanisms, which remains to be shown. In other yeasts amine oxidase, urate oxidase and D-amino acid oxidase have been found in peroxisomes (Veenhuis & Harder 1991). It would be worthwhile to look for these activities in peroxisomes of *S. cerevisiae* grown under optimal induction conditions. Identification of more peroxisomal proteins will broaden the experimental basis to investigate peroxisome biogenesis.

Peroxisomal mutants of S. cerevisiae

The recently described induction of peroxisomal proliferation in *S. cerevisiae* (Veenhuis et al. 1987), using oleic acid as sole carbon source, opened the possibility to dissect peroxisome biogenesis genetically and thus to identify components essential for this process. As a first step a screening procedure for cells with peroxisomal defects (Erdmann et al. 1989) was devised which resulted in mutants defective in a broad array of peroxisomal gene products (Höhfeld et al. 1992). Cells able to grow on acetate or glycerol but unable to grow on plates containing oleic acid as sole carbon source (oleic acid non-utilizing phenotype, *onu*-strains) were isolated. This screening procedure discriminated between colonies which cannot utilize oleic acid and those with other metabolic defects. Like other yeasts (Kunau et al. 1988) *S. cerevisiae* does not contain a mitochondrial β-oxidation system (Veenhuis et al. 1987). Therefore, the inability to grow on oleic acid can be used as a criterion for an impaired peroxisomal fatty acid degradation. In order to identify mutants with a defective β-oxidation among the *onu*-strains biochemical methods were applied. Such mutant phenotypes can be due to either defects in structural genes of β-oxidation enzymes (fox-mutants) or defects in peroxisomal assembly (pas-mutants). The determination of β-oxidation activities in either whole-cell lysates or subcellular fractions obtained from lysates of spheroplasts allow detection of deficiencies of individual enzyme activities as well as detection of all or only distinct peroxisomal matrix enzymes in the cytosol rather than in peroxisomes. This mislocalization of peroxisomal enzymes was taken as an indication that peroxisomes are absent or import-incompetent. These conclusions were further verified by electron microscopy and immunocytochemistry.

The fox-mutants fall into three complementation groups (FOX1-3) in agreement with the biochemical results that in peroxisomes three different proteins catalyze the four reactions of the β-oxidation cycle. An acyl-CoA oxidase (FOX1 or POX1, Dmochowska et al. 1990) converts the activated fatty acid to a corresponding α, β-transunsaturated enoyl-CoA ester. The second and third reaction is catalyzed by a multifunctional protein (FOX2) converting this unsaturated acyl-CoA ester to the corresponding β-oxoacyl-CoA ester. This metabolite, in turn, is cleaved by a thiolase (FOX3) resulting in an activated fatty acid, which is two carbon atoms shorter than the starting compound and again serves as substrate for the first reaction. An acyl-CoA oxidase gene (FOX1 or POX1) was cloned searching for a completely unrelated gene as an unidentified open reading frame. It was identified on the basis of its sequence similarity to the acyl-CoA oxidase genes POX4 and POX5 of C. tropicalis (Dmochowska et al. 1990). Functional complementation of fox2 and fox3 mutants using a genomic library of *S. cerevisiae* led to the characterization of the FOX2 (K. Hiltunen, manuscript submitted) and FOX3 genes (W.-H. Kunau et al. un-

publ.), respectively. All fox mutants possess morphologically normal peroxisomes.

There are other mutants with defects in genes encoding peroxisomal matrix enzymes: cta1, deficient in catalase A (Cohen et al. 1985), cit2, deficient in citrate synthase 2 (Rosenkrantz et al. 1986), and mdh2 deficient in malate dehydrogenase 2 (Minard & McAllister-Henn 1991). They were isolated using entirely different screening procedures specific for the individual enzymes and not with the intention of finding peroxisomal mutants. In all three cases the wild-type genes were cloned by functional complementation of the corresponding mutants.

Mutants with defects in structural genes of peroxisomal matrix proteins for which the phenotypical difference to wild-type strains allows the application of an assay system should be very valuable as *in vivo*-import systems. The possibility to transform them with plasmids carrying genes modified by *in vitro*-mutagenesis and to assay import by functional complementation make them at present an attractive alternative to *in vitro*-import systems (see below).

The pas-mutants are the second group of peroxisomal mutants of *S. cerevisiae* isolated besides the fox-mutants employing the above described screening procedure. This group comprises strains defective in peroxisome assembly and the mutants isolated so far fall into 13 complementation groups. Five additional complementation groups were found in the laboratory of H.F. Tabak (I. van der Leij et al., manuscript submitted). Mislocalization of peroxisomal matrix proteins to the cytosol was found for oleic acid-induced cells of all pas-mutants. The complexity of peroxisome assembly is not only reflected by the genetic diversity of the peroxisomal mutants but also by their different phenotypes representing three different classes. Mislocalization concerns either all (type I and type II) or only distinct peroxisomal matrix proteins (type III). Peroxisomes are normal in size and number (type III), present but non-proliferating (type II) or morphologically not detectable (type I) (for a detailed discussion see Höhfeld et al. 1992).

The pas-mutants of seven of our thirteen complementation groups were classified according to the three pas-types (Table 2). Absence or presence of few small peroxisomes can only be unambiguously decided by investigating tight phenotypes. For the remaining six complementation groups deletion mutants are not yet available. Therefore, it cannot be decided whether these mutants belong to type I or type II phenotype. Biochemical evidence excluded the type III phenotype.

Originally, we defined the pas-phenotype by the absence of morphologically detectable peroxisomes resulting in mislocalization of matrix pro-

Table 2. pas-mutants and PAS genes.

Complementation group	Number of alleles	Pastype	Corresponding wild type genes		
			Cloned	Sequenced	Features
PAS1	4	I	+	+	putative ATPase
PAS2	1	I	+	+	putative UBC[a]
PAS3	4	I	+	+	integral PMP[b]
PAS4	1	II	+	+	Zn-finger like motif required for proliferation
PAS5	1	I	+	+	Zn-finger like motif
PAS6	2	II(?)	+	+	C-terminal S-K-L
PAS7	2	III	+	+	required for thiolase import
PAS8	3	n.d.	−	−	
PAS9	1	n.d.	+	+	
PAS10	1	n.d.	−	−	
PAS11	1	n.d.	+	−	
PAS12	1	n.d.	+	−	
PAS13	1	n.d.	−	−	

[a] ubiquitin conjugating enzyme; [b] peroxisomal membrane protein.

teins and the inability to grow on oleic acid (type I) (Erdmann et al. 1989). However, detailed analysis of the peroxisomal mutants revealed that some of them possess morphologically recognizable peroxisomes (type II and III). These findings lead to a new definition of the pas-phenotype: common to all pas-mutants is the absence of functional peroxisomes resulting in (i) inability to grow on oleic acid and (ii) mislocalization of peroxisomal matrix enzymes. Their defects are not due to mutations in structural genes coding for metabolic enzymes (excluding e.g. fox-mutants, cta 1, cit2, and mdh2).

The isolation of pas-mutants allowed the use of functional complementation to identify genes encoding proteins essential for peroxisome biogenesis. Based on this approach the genes PAS1 to 7, 9, and 12 have been cloned in our laboratory (see Table 2). With the exception of PAS12 the complementing DNA-fragments have been sequenced and their open reading frames have been found to encode proteins not identical to any known protein. Four of the PAS gene products (Pas1p, Erdmann et al. 1991, Pas2p, F.F. Wiebel & W.-H. Kunau, in preparation, Pas4p, D. Mertens & W.-H. Kunau, in preparation, and Pas5p, A. Skaletz & W.-H. Kunau, unpubl.) exhibited at least partial sequence similarities to known proteins (see Table 2 and Höhfeld et al. 1992), one (Pas3p) contains features expected for a membrane bound receptor (Höhfeld et al. 1991) and one (Pas6p, A. Skaletz & W.-H. Kunau, unpubl.) contains the C-terminal putative peroxisomal targeting signal, Ser-Lys-Leu (see below). For all the other PAS gene products sequence analysis does not permit any conclusions regarding their function or intracellular location.

The further development of classical and molecular genetics in other yeasts, e.g. *P. pastoris* (Gleeson & Sudberry 1988) and *H. polymorpha* (Gleeson & Sudberry 1988, Cregg et al. 1990) increases the number of organisms suitable for genetic dissection of peroxisome biogenesis. This is emphasized by the isolation of peroxisomal mutants of *H. polymorpha* (Cregg et al. 1990; Didion & Roggenkamp 1990) and *P. pastoris* (J.M. Cregg & M. Veenhuis, pers. commun.). A genetic approach using appropriate mutants was also successful for the identification of a protein essential for peroxisome biogenesis in CHO-cells (Tsukamoto et al. 1991).

Import of peroxisomal proteins

Experimental approach

Peroxisomes, like mitochondria and chloroplasts, are thought to be formed from pre-existing organelles. Peroxisomal proteins are nuclear-encoded, synthesized on free polyribosomes and post-translationally imported into the organelles (for a review see Lazarow & Fujiki 1985). Most of them are synthesized at their final size, excluding major processing steps associated with import. Many peroxisomal functions as well as the import machinery seem to be conserved between organisms of different kind (Borst 1989). Heterologous peroxisomal proteins are imported into microbodies of different host cells including *S. cerevisiae* (Distel et al. 1987; Hansen & Roggenkamp 1989; Gödecke et al. 1989; Gould et al. 1990; McCammon et al. 1990b; Keller et al. 1991). However, peroxisomal targeting signals from proteins of *S. cerevisiae* have not yet been found experimentally. The main obstacle is the lack of an efficient *in vitro*-import system for peroxisomes, comparable to the system established for mitochondria (Hartl et al. 1987). With such a system the nature of the targeting signal(s) as well as the mechanism of import into peroxisomes could be investigated. The major advantages of the *in vitro*-import system for mitochondria are the existence of an assay for the quality of the organelle preparation (tight coupling of respiration and oxidative phosphorylation) and the fact that most mitochondrial proteins are made as larger precursors and are processed only inside the organelle, leaving no doubt whether the object of choice was at least partially translocated to the mitochondrial matrix or not. Compared to mitochondria, peroxisomes are very fragile organelles (Alexson et al. 1985). To date the most reliable peroxisomal *in vitro*-import system established is that for rat liver peroxisomes (Fujiki & Lazarow 1985). However, all *in vitro*-systems for peroxisomes are limited by

the fact that there are no definite criteria for the functional intactness of isolated organelles (see e.g. Bellion & Goodman 1987; Imanaka et al. 1987) not for import itself, since peroxisomal proteins are usually not processed. The exceptions to that rule, malate dehydrogenase of watermelon (Gietl 1990) and rat liver thiolase (Hijikata et al. 1987), have not been tried in an *in vitro*-system from yeast. Therefore, the only criterion that a polypeptide has been imported is the protection of this protein against proteases in the presence of a very fragile and fairly protease sensitive organelle. The small percentage of membrane associated or imported protein which is usually observed (Imanaka et al. 1987; Small et al. 1987, 1988; Miyazawa et al. 1989; Thieringer et al. 1991) does not allow a reliable conclusion whether the labeled protein has been translocated or not. As long as clear evidence for true import into peroxisomes *in vitro* is missing, all results based on this kind of experiments require confirmation by an independent method. Additionally, microbodies of *S. cerevisiae* seem to be more fragile than peroxisomes from other organisms. This difference, however, might be due to the procedures employed for induction and isolation of these organelles from *S. cerevisiae*.

In yeasts, especially in *S. cerevisiae*, targeting experiments can also be carried out *in vivo*, taking advantage of the easy manipulation of a unicellular eukaryote. However, in most cases, particularly when an assay system is not yet available, overexpression of peroxisomal proteins from multicopy vectors and/or strong promoters seems to be essential to even recognize the gene product of interest in the cell with antibodies. As far as we know the only exception to that is thiolase. This might be due to antibodies of extreme high affinity to this protein. For *in vivo* experiments transformed cells are grown in the presence of an inducer of peroxisomes and processed for analysis. In most cases the determination of the protein location occurs via immunological methods after fixation (for electron microscopy) or spheroplasting and immobilization (for immunofluorescence) or spheroplasting followed by gentle lysis, differential centrifugation and separation of organelles on a density gradient. The last method, although in principle suited to be performed in every laboratory might result in artefacts due to inclusion body formation after overexpression of proteins (Mitraki & King 1989; Hartig et al. 1990). Conclusions reached on the basis of immunofluorescence in *S. cerevisiae* cells (Pringle et al. 1989) have to take into account that the cell is very small and subcellular structures are at the limit of the optical resolution of a microscope. While it is possible to define the cytosolic or the nuclear location of a protein, in most cases the organellar location of a protein can only be characterized by punctuate appearance of the fluorescent dye, which means association with particles. Whether these are small protein aggregates or mitochondria or peroxisomes can usually not be distinguished by immunofluorescence despite the possibility of double labelling. Improvements of currently employed techniques of optical resolution, e.g. by confocal laser scanning microscopy, might result in more specific data. Certainly the best way to define the location of a protein inside a *S. cerevisiae* cell is the use of an electron microscope and immunocytochemistry. Numerous reports have been published using this method, and it would be beyond the scope of this review to give any details (for an example see van der Klei 1991 and Fig. 3).

Targeting signals for peroxisomal proteins can also be found by functional complementation of corresponding yeast mutants taking advantage of all the knowledge of the genetics and biochemistry of *S. cerevisiae*. However, not all imported proteins are active probably due to lack of oligomerization (Distel et al. 1987). An interesting case may be the use of the fox-mutants. Functional complementation of these mutants with enzymes of the β-oxidation pathway from higher eukaryotes including their mitochondrial counterparts will provide new insights into the mechanisms of β-oxidation including oligomerization and stereochemical aspects. In *S. cerevisiae* an additional approach for mechanistic studies on transport of proteins is available: the use of semipermeable cells (Baker et al. 1988) might prove very valuable also for import into peroxisomes. However, the lack of a reliable test system for import might represent a major obstacle in this case, too (see above).

Targeting signals

In higher eukaryotes the best defined targeting signal for peroxisomal proteins consists of a C-terminal tripeptide (S/A/C – K/R/H – L), which is necessary and sufficient to direct the proteins themselves and cytosolic reporter proteins into peroxisomes (Gould et al. 1987, 1989; Osumi & Fujiki 1990). Peroxisomal proteins of various organisms can be imported into peroxisomes of *S. cerevisiae* demonstrating the conserved nature of peroxisomal targeting (see above). However, not all reporter proteins (e.g. DHFR) with the short tripeptide added to their C-termini enter peroxisomes of *S. cerevisiae in vivo* (B. Distel, pers. commun.; A. Hartig et al., unpubl.). They obviously lack an additional important feature necessary for import into peroxisomes of *S. cerevisiae* but present in the peroxisomal proteins themselves. Whether this feature is a defined sequence or a certain structure allowing the presentation of either S-K-L or any other signal to its recognition factor (receptor) is still unknown yet. From a number of import experiments with a series of fusion proteins no conclusive evidence regarding a peroxisomal targeting signal in *S. cerevisiae* could be deduced (A. Hartig et al. unpubl.). These hybrid proteins consisted of fusions between DHFR or subunit IV of cytochrome oxidase and parts of catalase A or C-termini of various peroxisomal proteins. In addition, gene deletion- and fusion-studies with alcohol oxidase of *H. polymorpha* in *S. cerevisiae* did not reveal any umambiguous signal for translocation either (Distel 1990). Therefore, the notion mainly developed for higher eukaryotes that peroxisomal targeting signals consist of only short tripeptides at the C-terminus could not be verified for *S. cerevisiae* in this simple form. Moreover, not all peroxisomal proteins do contain an S-K-L or any of its known degenerated versions at the C-terminus (see Table 1). The idea that this signal tripeptide can also act at internal locations as suggested (Gould et al. 1989) is not supported by experimental evidence yet. In contrast, there is growing evidence that such a sequence at internal locations is dispensable for import into peroxisomes (J.M. Goodman, pers. commun.).

Among the pas-mutants one complementation group was found whose defect was identified as the mislocalization of thiolase but not of other matrix proteins (see above). This finding already suggests the existence of at least two different import pathways for peroxisomal matrix proteins, one for thiolase and another one for the other enzymes tested. In addition, the observation that there is no competition for import between catalase A and thiolase (M. Marzioch & W.-H. Kunau, unpubl.; A. Hartig et al., unpubl.) supports this idea. Furthermore, the targeting signal of rat peroxisomal thiolase was identified to reside on the N-terminus of this peroxisomal protein (Swinkels et al. 1990). Moreover, for the glyoxysomal malate dehydrogenase of watermelon an NH_2-terminal targeting signal has been suggested (Gietl 1990). However, for the peroxisomal malate dehydrogenase MDH2 of *S. cerevisiae* (Minard & McAllister-Henn 1991) no evidence for an amino-terminal transit peptide exists. In the light of these recent findings an earlier observation becomes interesting: In addition to the C-terminal targeting signal the firefly luciferase obviously contains also in the N-terminal region sequences or structures necessary for import into peroxisomes (Gould et al. 1987). Altogether, in *S. cerevisiae* and probably in other organisms, too, for some proteins the C-terminal amino acids are necessary for translocation into peroxisomes, but they may not be sufficient. For others, the extreme C-terminus does not seem to be involved in targeting at all.

Peroxisomal membrane proteins presumably are targeted to peroxisomes by a separate pathway. Current knowledge about biogenesis of peroxisomes (Lazarow & Fujiki 1985) leads to the proposal that peroxisomal prestructures exist in pas-mutant cells (for a detailed discussion see Höhfeld et al. 1992), resembling peroxisomal membrane ghosts found in Zellweger syndrome fibroblasts (Santos et al. 1988). This hypothesis is strengthened by the experimental finding that proliferation of membranes and induction of membrane proteins precedes the induction of matrix proteins (Veenhuis & Goodman 1990; Lüers et al. 1990).

Regulation of gene expression

Proliferation of microbodies in yeasts is repressed by glucose and induced by various carbon or nitrogen sorces (Tanaka & Fukui 1989; Veenhuis & Harder 1991). Therefore, it would not be surprising if most genes encoding peroxisomal proteins were regulated in the same way. However, analysis of gene regulation with respect to peroxisome biogenesis has been performed exclusively in *S. cerevisiae*, simply because the corresponding methods are well established for this organism alone. The best studied example is the CTA1 gene encoding the peroxisomal catalase A. Glucose repression of catalase A activity has been known for some time (Cross & Ruis 1978; Rytka et al. 1978) and was demonstrated to occur at the transcriptional level (M. Simon et al., manuscript submitted). The same is true for the FOX3-gene encoding peroxisomal β-ketothiolase (Einerhand et al. 1991), the FOX2-gene encoding the multifunctional protein of peroxisomal β-oxidation (K. Hiltunen et al., 1992; M. Simon et al., 1992), the PAS1 gene (Erdmann et al. 1991) and the PAS3 gene (Höhfeld et al. 1991). Four genes (CTA1, FOX2, FOX3 and PAS1) were shown to be derepressed on a non-fermentable carbon source and fully induced on oleic acid (Erdmann et al. 1991; Einerhand et al. 1991; M. Simon et al., 1992). The two key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, most probably also peroxisomal matrix enzymes (see above and Table 1) can be induced by oleic acid, too (McCammon et al. 1990a). Their activities can hardly be detected in cells grown on glucose (Duntze et al. 1969) and their regulation probably occurs on the transcriptional level.

In order to gain insight into the mechanisms regulating the expression levels of the genes mentioned above a molecular analysis of their promoter regions was started. Cis-acting regulatory DNA-elements and trans-acting factors were identified by comparing promoter sequences of genes encoding peroxisomal proteins with each other and with short sequences already known to be involved in regulation of gene expression. The promoter regions of the genes coding for the three β-oxidation enzymes, acylCoA-oxidase, multifunctional protein and thiolase, contain a short region of high similarity termed β-oxidation box and a similar sequence was identified in the promoter region of the CTA1 gene (Einerhand et al. 1991). It was demonstrated that this sequence of the thiolase promoter is responsible for mediating induced expression in the presence of oleic acid. A trans-acting factor binding to this newly identified cis-acting element has not been found yet. Upstream of that region in the FOX3-promoter a sequence was identified which was bound by the ARS binding factor *in vitro*. However, this element does not mediate a carbon source response *in vivo* (Einerhand et al. 1991). Similar experiments analyzing the CTA1-promoter resulted in the identification of an ADR1-binding site (Simon et al. 1991). ADR1, originally found to be necessary for derepression of the ADH2 gene (Denis & Young 1983), regulates the expression of the CTA1 gene as well as the expression of the genes FOX2, FOX3 and PAS1 (Simon et al. 1991). Moreover, using appropriate mutants two other genes, SNF1 and SNF4, known to control the derepression of glucose repressible genes (Carlson 1987), were identified to be involved in transcriptional regulation of the four genes CTA1, FOX2, FOX3 and PAS1 (M. Simon et al., 1992). These data suggest that defects in one of these trans-acting factors should lead to impaired peroxisome biogenesis. In fact, no peroxisomes could be detected in snf1- and snf4-mutant cells, and the peroxisomes observed in adr1-cells are small and not clustered like in wild type cells. Thus, it can be expected that genes encoding these and functionally similar proteins will be detected among the PAS genes. One example has already been found (PAS14 = SNF1, I. van der Leij et al., manuscript submitted) and with new mutant hunts using different screening procedures more such regulatory mutants might appear.

The results of these first few studies already suggest a very complex regulation of peroxisome biogenesis. The mode of action of the three trans-acting factors ADR1, SNF1 and SNF4 is different indicating the involvement of at least two separate signal pathways (M. Simon et al., 1992). It can be expected that more trans-acting factors will be identified which mediate specific and general me-

tabolic regulations of genes encoding peroxisomal proteins. Moreover, the sequence of events leading to inheritance and proliferation of peroxisomes needs also a complicated regulatory network. The studies described in this section mark only the beginning of our knowledge concerning gene expression related to peroxisomes.

Concluding remarks

In the yeast *Saccharomyces cerevisiae* the application of two genetic selections (Erdmann et al. 1989; H.F. Tabak, pers. commun.) has resulted in the isolation of a large number of mutants with defects in peroxisomal assembly (pas-mutants) and/or peroxisomal functions (fox-mutants). Among the pas-mutants 18 different complementation groups have been identified at present. Their diverse biochemical and morphological phenotypes resemble many of the phenotypes reported for fibroblasts of patients with peroxisomal disorders. These disorders are a newly recognized class of human inborn errors with severe clinical symptoms (Lazarow & Moser 1989). In most cases they are lethal. It is tempting to assume that the phenotypical similarities between cells of fungal peroxisomal mutants and cells of patients with peroxisomal disorders reflect deficiencies in components conserved in yeast and man. The yeast mutants have led to the isolation of the first PAS-genes which encode proteins essential for the biogenesis of peroxisomes in *S. cerevisiae*. Experiments are under way to find their counterparts in higher eukaryotes.

Another important challenge in the study of peroxisome biogenesis will be to understand which step the distinct PAS-proteins catalyze. Elucidation of mechanisms underlying human diseases is often facilitated through model systems. All the studies summarized in this review suggest that *S. cerevisiae* is such an experimentally tractable system.

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