A MOLECULAR GENETIC APPROACH TOWARD UNDERSTANDING PEROXISOME BIOGENESIS IN *HANSENULA POLYMORPHA*

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ABSTRACT

A MOLECULAR GENETIC APPROACH TOWARD UNDERSTANDING PEROXISOME BIOGENESIS IN *HANSENULA POLYMORPHA*

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Supervising Professor: James M. Cregg

This thesis describes the identification and characterization of *H. polymorpha* peroxisome-deficient mutants (*per* mutants) as well as the cloning and analysis of *PER8*, a gene required for peroxisome biogenesis in *H. polymorpha*. Methanol-utilization-defective mutants were subjected to electron microscopic examination and 38 of them were identified to be completely devoid of peroxisomes. Complementation analysis and genetic mapping demonstrated that the strains represented 11 different *PER* genes. Two strains were identified that harbored dominant-negative alleles. The remaining 36 strains contained recessive alleles. The expression and subcellular localization of peroxisomal matrix enzymes in these mutants were determined by differential centrifugation and immunocytochemical methods. Alcohol oxidase and catalase, peroxisomal marker enzymes, were demonstrated to be mislocalized to the cytosol in all mutants.

The *PER8* gene was cloned by functional complementation of the *per8* mutant and was localized to a 1.6-kb fragment by subcloning, northern blot analysis, and the DNA

sequencing of the gene. The sequence of *PER8* revealed an open reading frame of 885 bp that encoded a novel 295 amino acid polypeptide with a calculated molecular mass of 34 kD. Antibodies against Per8p recognized a protein of 31 kD. A mutant strain, in which a large portion of the *PER8* gene was deleted, was constructed and used to genetically demonstrate that the cloned gene was the same gene defective in original *per8* mutant. Per8p was shown to be an peroxisomal integral membrane protein that was concentrated in the membranes of newly formed organelles. Overexpression of *PER8* resulted in a markedly enhanced increase in peroxisome numbers. Thus, Per8p appears to play a role in peroxisome proliferation.

CHAPTER 1

INTRODUCTION

Eukaryotic cells are elaborately subdivided into functionally distinct membrane-bound compartments or organelles. To perform its specific set of metabolic functions, each organelle contains its own distinct set of proteins and other specialized molecules that must be properly sorted to that organelle. In addition, cells must assemble and maintain each of these organelles and assure the proper segregation of each component during cell division. Some organelles, like endoplasmic reticulum (ER), mitochondria, and vacuoles, have been extensively investigated (Schatz & Butow, 1983; Klionsky et al., 1990; Walter & Lingappa, 1986). Comparatively, little is known about these processes in peroxisomes.

Peroxisomes are ubiquitous eukaryotic organelles involved in a variety of important metabolic pathways (Tolbert, 1981; van den Bosch, 1992). The importance of peroxisomes in humans is demonstrated by the existence of a family of lethal genetic disorders called Zellweger syndrome in which peroxisomes appear to be absent (Goldfischer et al., 1973; Goldfischer, 1988). While peroxisomes are essential for the survival of humans as a whole organism, cell lines derived from Zellweger patients and peroxisome-deficient mutants of lower eukaryotes appear to be able to survive and grow without the organelles under certain nutritional conditions (Goldfischer, 1988; Cregg et al., 1990). Peroxisomes can be induced to proliferate in response to external stimuli and structurally, they are relatively simple compared to other organelles. Thus, peroxisomes may provide a good model system to understand basic aspects of organelle biogenesis. This thesis describes investigations on the molecular mechanisms of peroxisome

biogenesis in a methylotrophic yeast, *Hansenula polymorpha,* using a combination of genetic, biochemical, molecular biological, and cell biological approaches.

1.1 Definition of Peroxisomes

Peroxisomes were first described in mouse kidney cells by Rhodin in 1954 as small electron-dense vesicles bound by a single membrane and initially given the morphological name microbodies (Rhodin, 1954). Through cell fractionation and biochemical studies, de Duve and co-workers established that microbodies in higher organisms contain enzymes involved in the production and degradation of H_2O_2 (de Duve et al., 1960; de Duve & Baudhuin, 1966). Based on its role in H_2O_2 metabolism, the organelle was given the biochemical name peroxisome (Baudhuin et al., 1965). Today, peroxisomes and two other related organelles, the glyoxysomes in plants and fungi (Breidenbach & Beevers, 1967) and the glycosomes in certain parasites (Opperdoes & Borst, 1977), are referred to collectively as either microbodies or peroxisomes.

Peroxisomal matrix enzymes are involved in a number of important metabolic pathways but vary depending on the organism, tissue, stage of development and environmental conditions (Tolbert, 1981; van den Bosch, 1992). The only metabolic pathway common to all peroxisomes is the β-oxidation of fatty acids (Cooper & Beevers 1969; Lazarow & de Duve, 1976; Opperdoes, 1987). Peroxisomes can undergo rapid and dramatic changes in size, number, and enzyme content. In this section, we summarize the structure and function of peroxisomes and advances in the field of peroxisome biogenesis.

1.1.1 Peroxisomal respiration

The first peroxisomal function described by de Duve was respiration (de Duve et al., 1960; de Duve and Baudhuin, 1966). The peroxisomal respiration pathways consist

of at least one hydrogen peroxide (H_2O_2)-generating flavin oxidase and catalase (CAT) to degrade H_2O_2. These oxidases utilize molecular oxygen to directly oxidize a variety of substrates. The substrates for peroxisomal oxidases include lactate, glycolate and other α-hydroxy acids, urate, D-amino acids (de Duve and Baudhuin, 1966), acyl-CoA (Casteels et al., 1990), polyamines (Holtta, 1977), and oxalate (Beard et al., 1985). The decomposition of reactive and toxic H_2O_2 by CAT can occur by either of the two mechanisms. The first is a catalatic reaction that converts H_2O_2 into water and oxygen. Alternatively, H_2O_2 may be reduced to water by a peroxidatic reaction using a second substrate, such as alcohol, nitrites, formate or quinones, as a reductant (Chance & Oshino, 1971). The energy of the peroxisomal oxidation is dissipated as heat which contributes to thermogenesis. The compartmentation of H_2O_2 production and decomposition reduces the leakage of this toxic byproduct, and thus reduces potential cell damages (Chance & Oshino, 1971).

1.1.2 Lipid metabolism

1.1.2.1 β-oxidation

The presence of a nonmitochondrial β-oxidation system was first described in glyoxysomes from germinating castor bean endosperm (Cooper & Beevers, 1969). Subsequently, peroxisomal β-oxidation has been detected in animals (Lazarow & de Duve, 1976), protozoa (Blum, 1973), and eukaryotic microorganisms (Veenhuis et al., 1987). In this pathway, fatty acids are first converted to their corresponding acyl-coenzyme A (CoA) compounds by means of an acyl-CoA synthase, CoA, and ATP (Shindo & Hashimoto, 1978). The peroxisome has its own acyl-CoA synthases present on the peroxisomal membrane (Mannaerts et al., 1982; Bhushan et al., 1986). Following activation, acyl-CoAs are carried across the peroxisomal membrane and subjected to oxidation by acyl-CoA oxidase. H_2O_2 is formed in this first reaction and acyl-CoA is

oxidized to 2-enoyl-CoA. The next two reactions are catalyzed by a multifunctional enzyme that exhibits 2-enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase activities (Osumi & Hashimoto, 1979; Furuta et al., 1980). Lastly, acetyl-CoA and an acyl-CoA product that is shorter by two carbons are produced by a thiolytic cleavage activity of 3-oxoacyl-CoA thiolase (Miyazawa et al., 1981). All of these enzymes are located in the peroxisomal matrix. Although most β-oxidation reactions take place in mitochondria in animal cells, the peroxisomal pathway preferentially, if not exclusively, oxidizes specific types of fatty acids such as very long chain fatty acids (VLCFA, containing 24 carbons or greater) (Kawamura et al., 1981; Singh et al., 1984), long chain unsaturated fatty acids (Bremer & Osmundsen, 1984), branched chain fatty acids (Singh et al., 1990), long chain dicarboxylic acyl-CoAs (Mortensen et al., 1982), prostaglandins (Pace-Asciak & Granstrom, 1983) and certain xenobiotic compounds (Yamada et al., 1984). The significance of peroxisomal β-oxidation reactions in humans is demonstrated by certain inherited peroxisomal disorders including X-linked childhood adrenoleukodystrophy (X-ALD) and its phenotypic variants, in which VLCFAs accumulate in tissue and plasma due to a deficiency in VLCFA-CoA synthase (Arneson et al., 1982). X-ALD patients typically suffer from progressive destruction of cerebral white matter and adrenal cortex and usually die during adolescence. Unlike in mitochondria, fatty acids are incompletely oxidized by peroxisomal β-oxidation. Medium chain acyl-CoAs are exported via carnitine acyltransferase to the mitochondria for further oxidation (Farrell & Bieber, 1983). Thus, peroxisomal β-oxidation of these fatty acids becomes a mechanism by which poorly oxidizable fatty acids are converted to metabolically more palatable products.

1.1.2.2 Lipid biosynthesis

Peroxisomes are not only lipid-degrading organelles; they also participate in the biosynthesis of certain classes of lipids, specifically plasmalogen, cholesterol and bile acids (for review see Lazarow & Moser, 1989). Plasmalogens are a class of ether-linked phospholipids that are major components of cell membranes, especially those in nerve tissue. However, the function of plasmalogens is not fully understood. The first three steps of plasmalogen synthesis, involving an unusual exchange of an acyl group on C-1 of the phospholipid with a long-chain fatty alcohol, occur in peroxisomes (Hajra et al., 1979; Hajra & Bishop, 1982). The chief enzymes are dihydroxyacetone phosphate (DHAP) acyltransferase, alkyl-DHAP synthase and acyl/alkyl-DHAP reductase. All these enzymes are bound to the inside membrane of the peroxisome. Subsequent reactions in ether lipid biosynthesis occur in the ER.

Cholesterol is excreted from the body largely in the form of water-soluble bile acid conjugates. Bile acids are formed from cholesterol by hydroxylation of the ring structure and by β-oxidation of the cholesterol side chain (Bjorkhem, 1985). At least one of the hydroxylation pathway reactions and all of the β-oxidation reactions are peroxisomal (Krisans et al., 1985; Pedersen & Gustafsson, 1980). Peroxisomes have also been shown to contain the bile acid conjugation enzymes (Kase & Bjorkhem, 1989).

Three-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, the key regulatory enzyme in the biosynthesis of cholesterol and dolichol which regulate membrane stability, fluidity and permeability, is found in the peroxisomal matrix as well as in the ER membrane (Brown & Goldstein, 1980; Keller et al., 1985, 1986). In addition, peroxisomes contain other enzymes involved in cholesterol synthesis, such as thiolase, mevalonate kinase (Thompson & Krisans, 1990; Stamellos et al., 1992), dihydrolanosterol oxidase, steroid-3-ketoreductase, steroid-14-reductase, and steroid-8-isomerase (Appelkvist et al., 1990). Other proteins thought to be involved in cholesterol

metabolism, such as sterol carrier protein-2 (SCP-2) and apolipoprotein E (Apo E), are also present in peroxisomes (Keller et al., 1989; Hamilton et al., 1990). Although these enzymes are also located in the ER or cytosol, the low serum cholesterol levels in patients suffering from peroxisomal deficiency diseases and the reduced cholesterol synthetic capacity in cells lacking peroxisomes suggest that peroxisomes contribute to normal cholesterol levels (Scotto et al., 1982; Barth et al., 1987). Thus, by controlling cholesterol biosynthesis or degradation or both, peroxisomes may be critical in regulating human cholesterol levels (Thompson et al., 1987).

1.1.3 Glyoxylate cycle and photorespiration

The glyoxylate pathway provides a mechanism for the net conversion of fatty acids to carbohydrates in microorganisms and germinating fatty seeds (Beevers, 1975; Tolbert, 1981). This conversion is carried out by malate synthase and isocitrate lyase along with enzymes of the tricarboxylic acid cycle. All the enzymes of glyoxylate pathway were shown to be located in the glyoxysome, a specialized peroxisome in fatty seeds of certain plants (Breidenbach & Beevers, 1967; Cooper & Beevers, 1969b). The two key glyoxylate bypass enzymes, malate synthase and isocitrate lyase (but not the other enzymes of the glyoxylate cycle), are present in peroxisomes or glyoxysomes in yeast (Fukui & Tanada, 1979; Maxwell et al., 1977), *Tetrahymena* (Hogg, 1969), *Euglena* (Graves & Becker, 1974) and various fungi (Maxwell et al., 1977). The remaining enzymes are present in mitochondria. While animal cells are generally thought not to have the glyoxylate pathway, it has been reported that isocitrate lyase and malate synthase are present in several vertebrate tissues such as rat liver (Davis et al., 1989).

In photosynthetic tissues, the leaf peroxisome plays a major role in the conversion of glycolate to glycine in the oxidative photosynthetic carbon cycle of photorespiration (Tolbert & Yamazaki, 1969). Photorespiration involves the consumption of oxygen and

the formation of carbon dioxide and competes with photosynthetic carbon dioxide fixation (Larson & Kershaw, 1976). In photorespiration, ribulose 1,5-bisphosphate carboxylase (RuBP carboxylase-oxygenase or Rubisco), the carbon-fixing enzyme in photosynthesis, acts as an oxygenase and uses oxygen instead of carbon dioxide as a substrate. Glycolate produced by the chloroplast enters the peroxisome and is converted to glycine, which is exported to the mitochondrion for further metabolism. In some plants, photorespiration may occur at 25-50% of the rate of photosynthesis. The net result is that photorespiration dissipates carbon dioxide fixation and consumes energy (Ogren, 1984).

1.1.4 Alcohol metabolism

A few methylotropic yeast species, such as *H. polymorpha*, *Candida boidinii* and *Pichia pastoris,* are able to utilize methanol as the sole carbon source and energy source (Anthony, 1982; Veenhuis et al., 1983). Growth on methanol results in the induction of a unique set of pathway enzymes including alcohol oxidase (AOX), CAT and dihydroxyacetone synthase (DHAS), which are located in the peroxisomal matrix (Veenhuis et al., 1976, 1983; Douma et al., 1985). The pathway for methanol metabolism and compartmentation is shown in Figure 1.1. Methanol is first oxidized to formaldehyde by AOX, with H_2O_2 generated as a byproduct and then broken down to water and oxygen by CAT. Formaldehyde can be metabolized either for energy generation or for the synthesis of biomass. In the energy-generation pathway, formaldehyde exits to the cytosol and is oxidized to formate and then to carbon dioxide by two dehydrogenases which simultaneously reduce NAD to $NADH_2$. To generate biomass, formaldehyde enters a cyclic assimilatory pathway, the xylulose 5'-phosphate (Xu5P) cycle. The first enzyme of the pathway, DHAS, is peroxisomal and condenses formaldehyde with Xu5P to generate two C_3 compounds, glyceraldehyde-3-phosphate and dihydroxyacetone. These two compounds then leave the peroxisome and are metabolized by a series of

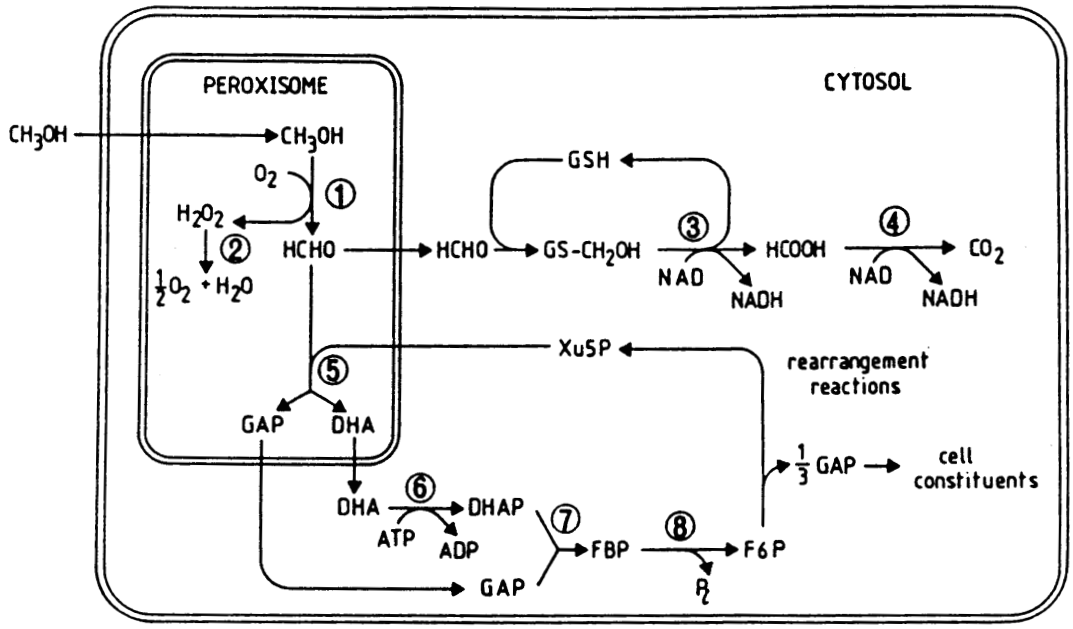


Figure 1.1 The methanol pathway in *Hansenula polymorpha*. 1. alcohol oxidase; 2. catalase; 3. formaldehyde dehydrogenase; 4. formate dehydrogenase; 5. dihydroxyacetone synthase; 6. dihydroxyacetone kinase; 7. fructose 1,6-bisphosphate aldolase; 8. fructose 1,6-bisphosphate phosphatase. (From Douma et al., 1985).

cytosolic enzymes that eventually regenerate Xu5P and generate one molecule of glyceraldehyde-3-phosphate for every three turns of the cycle. In addition, growth of all yeast species on C_2 carbon sources, such as ethanol, acetate and acyl-CoA, requires the peroxisomal glyoxylate-pathway enzymes isocitrate lyase and malate synthase acting together with enzymes of the citric acid cycle (Zwart et al., 1983).

In animals and humans, peroxisomes are involved in ethanol clearance, especially when ethanol is present at high levels (Oshino et al., 1975). Although most ethanol is oxidized by the cytosolic alcohol dehydrogenase, some is oxidized by the peroxidatic reaction of CAT in peroxisomes.

1.1.5 Other reactions

1.1.5.1 Glycolytic pathway

Glycolysis is nearly a universal pathway in biological systems for energy production. Some organisms like *Trypanosoma brucei* are entirely dependent on glycolysis for their production of energy. In *T. brucei*, seven enzymes involved in glycolysis are located in the glycosome, a specialized peroxisome (Opperdoes & Borst, 1977). Typical peroxisomal oxidation reactions such as β-oxidation and plasmalogen synthesis are also found in this organelle. The glycosomal membrane is thought to act as a permeability barrier which prevents the dilution of the glycolytic intermediates and cofactors, thereby effectively increasing their concentration and the rate of glycolysis. Glycosomes of certain *Trypanosomatides* lack a H_2O_2-producing oxidase and CAT, but those of related organisms do have CAT (Oppendoes, 1987).

1.1.5.2 Transamination

Conversion of amino acids to glucose begins with deamination which occurs in peroxisomes in humans (Hsieh & Tolbert, 1976). Human liver

peroxisomes contain two aminotransferases capable of transferring amino groups from certain amino acids to glyoxalate or pyruvate, yielding glycine or alanine, which enter the gluconeogenic pathway (Noguchi & Takada, 1978, 1979). Such transaminases include serine:pyruvate aminotransferase and alanine:glyoxalate aminotransferase (AGT). Gluconeogenesis from amino acids is important both on a high-protein diet and during starvation, when tissue protein is utilized. Hyperoxaluria type I, a serious human genetic disease, is caused by deficiency in AGT activity or by mislocalization of AGT to mitochondria (Danpure et al., 1986; Wanders et al., 1990).

1.2 Human Peroxisomal Disorders

The importance of peroxisomes in humans is demonstrated by a number of genetic disorders affecting peroxisomes (Moser et al., 1991; van den Bosch, 1992). These diseases can be divided into three groups depending on the degree to which peroxisomal functions are deficient (Godfischer, 1988; van den Bosch, 1992). The first group includes Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), infantile Refsum disease (IRD) and hyperpipecolic acidemia (HPA). Normal peroxisomes are missing from cells of patients with these diseases, but peroxisomal ghosts which consist of membrane and integral membrane proteins are found (Wiemer et al., 1989). In these cells, many peroxisomal enzymes are missing due to degradation and others are mislocalized to the cytosol. In the second group, a small subset of peroxisomal enzymes are defective. This group includes rhizomelic chondrodysplasia punctata (RCDP) and Zellweger-like syndrome. The third group comprises diseases in which only a single peroxisomal enzyme is deficient (Schram et al., 1986). These diseases include X-linked adrenoleukodystrophy (Arneson et al., 1982), acatalasemia, amyotrophic lateral sclerosis

(Lou Gehrig's disease) (McNamara et al., 1993; Rosen et al., 1993) and deficiencies in individual β-oxidation enzymes.

1.3 Peroxisome Biogenesis

It is now generally accepted that peroxisomes, at least under conditions of their proliferation, arise by growth and division from pre-existing peroxisomes (Lazarow & Fujiki, 1985; Borst, 1989; Subramani, 1993) rather than by budding from ER or *de novo* (Novikoff & Shin, 1964). In response to external stimuli, peroxisomes can undergo rapid and dramatic changes in size, number and enzyme content. The molecular mechanism(s) involved in integration of lipids into the peroxisomal membrane, the import of specific proteins into the peroxisome, the growth and division of the organelle and its segregation into daughter cells at cell division, is mostly unknown.

Peroxisomes contain no DNA (Kamiryo et al., 1982) and thus their formation is most likely under control of nuclear genes. All peroxisomal proteins, including matrix proteins and integral membrane proteins, are synthesized on free ribosomes in the cytosol, usually at their mature size (Goldman & Blobel, 1978; Roa & Blobel, 1983), and post-translationally imported into the organelle (Fujiki et al., 1984; Koster et al., 1986). This leads to progressive enlargement of the peroxisomes. Translocation of proteins into the organelle appears to require ATP hydrolysis (Nicolay et al., 1987), but not the proton gradient that exists across the peroxisomal membrane (Imanaka et al., 1987; del Valle et al., 1988; Douma et al., 1990; Wendland and Subramani, 1993).

1.3.1 Protein import into peroxisomes

Although the molecular mechanisms involved in the recognition by and incorporation of proteins into peroxisomes have not been elucidated, at least two distinct

targeting signals used by peroxisomal matrix proteins have been identified (for reviews see de Hoop & AB, 1992; Subramani, 1993). The most commonly utilized peroxisomal targeting signal (PTS1) is a carboxyl-terminal tripeptide sequence (SKL) and several conservative variants (Gould et al., 1987, 1988, 1989; Keller et al., 1987). PTS1 has been shown to be sufficient to direct cytosolic proteins to the organelle (Gould et al., 1989; Fung & Clayton, 1991; Swinkel et al., 1992). Furthermore, the sequence is present at the carboxyl-terminus of a number of peroxisomal matrix proteins in higher and lower eukaryotes (Gould et al., 1990a; Keller et al., 1991). Antibodies raised against the SKL tripeptide were able to recognize matrix proteins from peroxisomes in mammals, glyoxysomes in germinating seeds and glycosomes in *T. brucei* (Gould et al., 1990b; Keller et al., 1991). Thus, the SKL motif serves as a general microbody targeting signal and supports the common evolutionary origin of these organelles.

Unlike most peroxisomal proteins, the two rat peroxisomal thiolases (A and B) and watermelon malate dehydrogenase contain 36, 26 and 37 amino-terminal presequences, respectively, that are cleaved after import of the proteins into peroxisomes by a peroxisome-specific protease (Bodnar & Rachubinski, 1990; Gietl, 1990; Hijikata et al., 1990). As shown for PTS1, these presequences are sufficient to direct non-peroxisomal proteins to the organelle and therefore, are a PTS (PTS2) (Swinkels et al., 1991; Gietl et al., 1994). By deletion analysis, PTS2 is located within the first 11 amino acids of the presequence of thiolase B. *Saccharomyces cerevisiae* thiolase is similar in sequence to that of rat PTS2 at its amino-terminus and acts as a PTS, but is not cleaved after import (Swinkels et al., 1991). Analogous sequences are also found in thiolases from *Candida tropicalis* and *Yarrowia lipolytica* and other peroxisomal enzymes, including amine oxidase from *H. polymorpha* and aldolase from *T. brucei* (Clayton, 1985; Bruinenberg et al., 1989). The amino-terminus from aldolase has been shown to be a targeting signal for glycosome import (Blattner et al., 1991). Mutants defective in the transport of PTS1- but

not PTS2- containing proteins or vice versa have been reported in *P. pastoris* (McCollum et al., 1993), *S. cerevisiae* (Erdmann & Kunau, 1992; van der Leij et al., 1992) and certain Zellweger syndrome fibroblasts (Balfe et al., 1990; Motley et al., 1994). However, most peroxisome-deficient mutants in all organisms appear to be defective in the import of all matrix proteins. This suggests that the PTS1- and PTS2-dependent import machineries have at least one component in common.

Not all peroxisomal matrix proteins appear to have PTS1 or PTS2 sequences. One such protein is acyl-CoA oxidase from *C. tropicalis*. Two independent internal regions in acyl-CoA oxidase were identified by Small et al. (1988) to act as a PTS. Neither of these peptide segments resembles PTS1 or PTS2. Similarly, catalase A from *S. cerevisiae* has been reported to contain a carboxyl-terminal PTS similar to PTS1 and an independent internal PTS (Kragler et al., 1993). Therefore, additional peroxisomal PTSs are likely to exist. In addition, none of the peroxisomal membrane proteins (PMPs) described to date appear to have either PTS1 or PTS2. Furthermore, anti-SKL antibodies recognize many matrix proteins but not PMPs (Keller et al., 1991). Finally, fibroblasts from Zellweger syndrome patients appear to properly incorporate PMPs into peroxisomal ghosts (Santos et al., 1988; Gartner et al., 1991; McCollum et al., 1993). An internal region of the PMP47 has been shown to be essential for sorting to peroxisomes (McCammon et al., 1994). Therefore, it is likely that PMPs are targeted to the organelle using signal(s) and mechanism(s) that are different from those for matrix proteins.

Import of proteins into peroxisomes has been divided into two steps *in vitro*: an initial binding of protein to the peroxisomal membrane that is followed by its translocation into the peroxisomal matrix (Imanaka et al., 1987; Wendland & Subramani, 1993). The *PAS8* gene product from *P. pastoris* has been shown to specifically bind SKL-containing peptides and therefore may be a putative PTS1 import receptor (McCollum et al., 1993).

Biochemical evidence suggests that import requires both cytosolic factors as well as N-ethylmaleimide-sensitive membrane-associated proteins (Wendland & Subramani, 1993).

1.3.2 Peroxisomal membranes

Peroxisomal membranes are unusual in that they are relatively thin and appear to lack large proteinaceous structures (Veenhuis & Harder, 1988). The polypeptide composition of peroxisomal membranes is unique relative to that of other cell membranes and changes in response to environmental stimuli (Goodman et al., 1986). In contrast, their phospholipid composition is similar to that of other cellular membranes except for a low sterol content (Lazarow, 1984; Beevers & Gonzalez, 1987; Sulter et al., 1993a). Like those of other organelles, peroxisomal phospholipids are thought to be synthesized by the ER (Veenhuis & Harder, 1987; Lazarow, 1989). In many organisms and tissues, physical associations between developing (but not mature) peroxisomes and strands of the ER have been observed (Sulter et al., 1993a; Chapter 3, this thesis). In addition, ER proliferation is often seen under conditions of peroxisome induction in yeast (Veenhuis & Harder, 1987). These peroxisome-ER associations may function in the transfer of lipids.

1.3.3 Peroxisome induction

One striking property of peroxisomes is their ability to proliferate upon induction (Veenhuis et al., 1979; Lock et al., 1989). Proliferation is an adaptive response to changes in the external environment. In methylotrophic yeasts such as *P. pastoris*, *H. polymorpha*, and *C. boidinii*, peroxisomes increase enormously in size and number in methanol-grown cells (up to 80% of cell volume) compared to glucose-grown cells in which one or a few small peroxisomes exist (1% of the cytoplasmic volume) (Veenhuis et al., 1983). Induction results in the growth of the organelle by the import of peroxisomal proteins followed by proliferation of the organelle by budding from mature peroxisomes

(Lazarow et al., 1982). In addition, methanol induction results in the massive synthesis of key enzymes of the methanol pathway: AOX, CAT, and DHAS. Other compounds such as primary amines (amine oxidase), D-amino acids (D-amino acid oxidase) and purines (urate oxidase) can induce peroxisome proliferation in *H. polymorpha* (Veenhuis, 1992). In *S. cerevisiae* and *P. pastoris*, growth on fatty acids as the sole carbon source is sufficient to cause induction of peroxisomes containing enzymes of β-oxidation pathway (Veenhuis et al., 1987; Liu et al., 1992).

In rats, administration of certain hypocholesterolemic drugs such as clofibrate or some xenobiotic compounds (referred to as peroxisome proliferators) leads to a five- to nine-fold proliferation in peroxisome and an induction of specific peroxisomal enzymes (Reddy & Lalwani, 1983; Lazarow & Fujiki, 1985). These enzymes include carnitine acetyltransferase, carnitine octanoyltransferase, acyl-CoA:dihydroxyacetone phosphate acyltransferase and enzymes of the β-oxidation pathway, but not CAT (Lock et al., 1989). Clofibrate and similar drugs were investigated as potential therapeutics to reduce cholesterol levels in patients with ischemic heart disease (Havel & Kane, 1973). Unfortunately, the drugs also stimulate the induction of carcinomas in rat and mouse liver cells (Reddy & Qureshi, 1979). Since the drugs do not interact with or damage DNA directly, it appears that the hepatocarcinogenic activity may arise from the increased levels of peroxisomal oxidative reactions and the DNA-damaging activated-oxygen species that result due to low levels of CAT activity. In addition, clofibrate may also induce β-oxidation pathway enzymes in yeasts (van den Bosch, 1992). Thus, regulation of peroxisomal enzyme contents is important to cell survival.

A key feature of the molecular mechanism controlling peroxisome proliferation in mammals has been elucidated and is similar to that described for response to steroid hormones (Issemann & Green, 1990; Green, 1992). Activation appears to be mediated through distinct ligand-activated receptors, collectively known as peroxisome proliferator-

activated receptors (PPARs). Three different PPARs (α, β and γ) from *Xenopus laevis* (Dreyer et al., 1992), 9-cis retinoid acid-responding retinoid X receptor-α (RXRα) (Mangelsdorf et al., 1990) and PPARs from mouse (Issemann & Green, 1990) have been cloned. These receptors are members of the soluble receptor superfamily of transcriptional activators and consist of a transactivating domain, a ligand-binding domain and a putative zinc-finger DNA-binding domain. The peroxisome proliferators activate transcription by binding to PPARs which in turn bind to peroxisome proliferation-responsive promoter elements (PPREs) (Dreyer et al., 1992). PPREs have been identified upstream of several peroxisome-proliferator-inducible genes including rat hydratase-dehydrogenase (Zhang et al., 1993), rat acyl-CoA oxidase (Tugwood et al., 1992) and rat enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (Zhang et al., 1992a).

1.4 Approaches to the Study of Peroxisome Biogenesis

1.4.1 Biochemical approaches

1.4.1.1 *In vitro* import using purified peroxisomes

In vitro import systems have been demonstrated to be powerful tools to investigate the molecular mechanisms of targeting proteins to organelles such as the mitochondrion and the ER (Gasser & Schatz, 1983). The import of radiolabeled proteins into purified rat liver or yeast peroxisomes has been monitored by binding and proteinase protection assays (Imanaka et al., 1987; Small et al., 1987; Lazarow et al., 1991). The process was shown to be signal-, time-, energy- and temperature-dependent (Imanaka et al., 1987; Small et al., 1987). However, limitations have hampered this approach. Peroxisomes are very fragile and difficult to purify. In addition, reliable biochemical hallmark of peroxisomal protein import (such as signal cleavage or oligosaccharide addition used to study translocation into and transport through the ER) does not yet exist.

Import of proteins into purified peroxisomes is inefficient. *In vivo*, proteins appear to be imported only into newly formed peroxisomes while *in vitro* systems have typically used mature and possibly import incompetent peroxisomes (Veenhuis et al., 1992). Although much effort has been put into the development of such *in vitro* import systems, no reproducible system has emerged.

1.4.1.2 Microinjection

To bypass the limitations of *in vitro* import system based on purified peroxisomes, a microinjection system has been developed by Walton et al. (1992). Luciferase (containing PST1) or HSA-SKL (human serum albumin-SKL peptide conjugate) have been microinjected into mammalian cells and found to be properly imported into peroxisomes in a time-, signal- and temperature-dependent manner. Furthermore, the import of these proteins was only observed in normal human fibroblast cell lines but not in cell lines derived from Zellweger syndrome patients. The import of the PTS1 directed proteins could be inhibited by co-injection of an excess of synthetic peptides harboring Ser-Lys-Leu COOH, indicating that import was saturable. Similar results were obtained with *P. pastoris* AOX (which is thought to harbor a PTS1) except that microinjected AOX was imported in both cell lines. Thus, microinjection appears to provide a means to assay peroxisomal protein import in mammalian cell lines and especially cell lines derived from Zellweger syndrome patients. Variations on this approach should make it possible to elucidate the role of specific peroxisomal gene products in import as well.

1.4.1.3 Semi-intact cell systems

Recently semi-intact cell methods have been applied to study peroxisomal protein import (Rapp et al., 1993; Wendland & Subramani, 1993). Chinese hamster

ovary (CHO) cells were permeabilized by bacterial toxin streptolysin O (SLO) under conditions that retain the functional integrity of the peroxisomal membrane. The import of luciferase and HSA-SKL was observed by indirect immunofluorescence and found to be dependent on time, temperature, signal, ATP and cytosol. The advantage of the permeabilized cell system is that it allows the investigator to test and purify specific import factors. For example, it was shown that import was dependent on one or more cytosolic factors.

1.4.2 Genetic approaches

1.4.2.1 Peroxisome-deficient mutants in mammalian cells

Human Cells. Genetic methods have only recently been utilized to investigate peroxisomes. The first description of a genetic defect in peroxisome biogenesis was Zellweger syndrome (for details, see Section 1.2) (Goldfischer, 1973). In this disorder, most newly synthesized peroxisomal matrix enzymes are mislocalized to the cytosol, (e.g., CAT, D-amino acid oxidase and L-α-hydroxyacid oxidase), where many of them are rapidly degraded (e.g., acyl-CoA oxidase, multifunctional enzymes of β-oxidation pathway, DHAP acyl-transferase and alkyl-DHAP synthase) (Schram et al., 1986; for review see Lazarow and Moser, 1989). As a consequence, the corresponding peroxisomal functions, such as catabolism of very long chain fatty acids, pipecolic acid and phytanic acid degradation, bile acid formation, and biosynthesis of cholesterol and plasmalogens, are impaired (Schutgens et al., 1986). Cytologically, Zellweger patient cells are devoid of morphologically recognizable peroxisomes (Goldfischer, 1973). However, immunocytochemical studies have revealed the presence of nearly empty membrane-bound vesicles called ghosts (Santos et al., 1988; Wiemer et al., 1989). By using genetic complementation analysis, at least nine complementation groups have been identified (Lazarow & Moser, 1989; Shimozawa, 1992b; Lazarow, 1993). Detailed

examination of patient cell lines revealed that some complementation groups showed a general loss of PTS1 and PTS2 import while others were deficient in the import of PTS1 but not PTS2 or vice versa (Motley et al., 1994).

CHO Cells. CHO cell mutants defective in peroxisome biogenesis were initially isolated by screening for deficiency in the peroxisomal plasmalogen biosynthetic enzyme DHAP acyl-transferase (Zoeller et al., 1986). Three mutant lines lacking DHAP acyl-transferase were identified. The mutants are devoid of normal peroxisome structures and CAT is located in the cytosol. Immunoblot analysis demonstrated that peroxisomal proteins in the mutant CHO cells are deficient in a pattern similar to those in Zellweger syndrome. Three complementation groups of CHO peroxisome-deficient mutants have been reported (Tsukamoto et al., 1990; Shimozawa et al., 1992a).

1.4.2.2 Peroxisome-deficient mutants in yeast

Yeast has been a popular model system due to the ability to combine genetic and biochemical approaches toward problems in eukaryotic cell biology, especially those of organelle biogenesis. For peroxisome studies, yeast is particularly attractive since the proliferation and enzymic composition of peroxisomes can be readily manipulated by varying growth conditions (Veenhuis & Harder, 1989). Peroxisome-deficient yeast mutants have been reported in four yeast species: *S. cerevisiae*, *H. polymorpha*, *P. pastoris* and *Y. lipolytica* (Erdmann et al., 1989; Cregg et al., 1990; Gould et al., 1992; Liu et al., 1992; Nuttley et al., 1993). These yeasts have provided ideal conditionally-lethal mutants that are healthy under permissive conditions (e.g., glucose and ethanol) but totally inviable under nonpermissive conditions (e.g., methanol and/or oleate).

S. cerevisiae. In fungi, β-oxidation of fatty acids occurs exclusively in the peroxisome. The first peroxisome-deficient mutants (*pas* mutants) in this yeast were reported by Erdmann et al. (1989), based on the fact that peroxisomal β-oxidation as well

as peroxisome proliferation can be induced by oleic acid. The mutants were isolated by screening a collection of oleic acid non-utilizing strains for the absence of peroxisomes by electron microscopy. Four of 32 oleate non-utilizing mutants were identified that lack recognizable peroxisomal structures. Subcellular fractionation studies of two of these strains indicated that the peroxisomal β-oxidation pathway enzymes, CAT and 3-oxoacyl-CoA thiolase, were cytosolic. Further screening of *pas* mutants by this approach revealed at least 12 different complementation groups (Kunau and Harting, 1992). The complexity of peroxisome biogenesis is not only reflected by the number of *PAS* genes, but also by their different phenotypes. Three different subgroups of *pas* mutants, designated type I to III, have been distinguished. Type I *pas* mutants (*pas1, 2, 3, 5*) are characterized by the total absence of morphologically detectable peroxisomes. Peroxisomal matrix enzymes are present at wild-type levels but are not sedimentable. These mutants are thought to be affected in peroxisome formation. Type II *pas* mutants (*pas4, 6*) contain one or a few small peroxisomes upon oleic acid induction, a phenotype similar to those seen in glucose-repressed cells. Like type I mutants, peroxisomal matrix enzymes are present at normal levels and mostly mislocalized to the cytosol. However, a small portion of enzymes are correctly localized, indicating that these peroxisomes are import-competent. These mutants appear to be defective in proliferation. Type III mutants are defective in import of only one or a few individual peroxisomal proteins. *pas7* is defective only in 3-oxoacyl thiolase import and has normal-appearing peroxisomes. Thus, this mutant seems to affect a component essential for the import of PTS2-containing proteins. Recently, positive selection procedures for peroxisome-deficient mutants have been reported (Elgersma et al., 1993). A chimeric gene was constructed encoding bleomycin-resistant protein linked to peroxisomal luciferase. Wild-type cells in which this chimeric protein is imported into the peroxisome are phleomycin sensitive, while the *pas* mutants are phleomycin insensitive.

Additional *pas* mutants, including those representing three novel complementation groups, were identified.

H. polymorpha. *H. polymorpha* is a methanol-utilizing yeast whose growth on methanol is accompanied by massive peroxisome proliferation and induction of methanol-pathway enzymes. Peroxisome-deficient mutants in this strain (*per* mutants) were first reported by Cregg et al. (1990). Since both methanol and ethanol metabolism require peroxisomal enzymes, mutagenized cultures of the yeast were first screened for mutants that failed to utilize both alcohols as sole carbon source (Eut^-Mut^-). Two *per* mutants were identified by EM examination from the collection. Both mutants lacked recognizable peroxisomes and all peroxisomal enzymes examined were present but mislocalized to the cytosol. Subsequent backcrossing results revealed that Mut^- and Eut^- phenotypes segregated independently of each other and that the Per^- (peroxisome-less) phenotype co-segregated with the Mut^- phenotype. Thus, growth on ethanol does not need functional peroxisomes.

Additional *per* mutants were identified from a collection of 260 Mut^- mutants (Waterham et al., 1992; Titorenko et al., 1993; this thesis). Based on peroxisome appearance in methanol-induced mutant cells, three distinct phenotypic classes could be distinguished (Veenhuis et al., 1992). In the first class, peroxisomes appear completely absent (Per^- phenotype). Like Type I mutants of *S. cerevisiae*, peroxisomal enzymes are expressed and cytosolic. Peroxisomal membrane proteins (PMPs) are also normally induced but are located in small proteinacous/phospholipid aggregates. Under methanol-induced conditions, a large cytosolic crystalloid invariably developed in each mutant that was readily observed by light microscopy and was primarily composed of active AOX. Per^- collections represented mutations in 11 different *PER* genes. Two of the genes, *PER5* and *PER7,* were represented by both dominant-negative and recessive alleles. Peroxisomal import mutants (Pim^- phenotype), a second class, is similar to the Type II

mutants of *S. cerevisiae* morphologically and biochemically. Pim$^-$ mutants, like Per$^-$ mutants, also contain the large cytosolic AOX crystalloid. These mutants have been organized into five different complementation groups. The third phenotypic class of *per* mutants shows normal induction of peroxisome proliferation, but the peroxisomes harbor an aberrant crystalline matrix substructure (Pss$^-$ phenotype). It is speculated that the defect in these mutants may be in AOX assembly or transport of FAD. In addition, a series of conditional mutants, including temperature-sensitive (ts) mutants, pH-sensitive mutants, and cold-sensitive (cs) mutants, have been isolated. Detailed genetic analysis showed that the total *per* mutant collection represented mutations in 14 different genes (*PER1-14*) and that the three phenotypes were sometimes allele-specific.

P. pastoris. *P. pastoris* is unique in that it can grow on either methanol or oleic acid as a sole carbon source. An efficient screening procedure was devised and peroxisome-deficient mutants (*per* or *pas* mutants) were reported by Liu et al. (1992) and Gould et al. (1992). As a first step, strains that were Mut$^-$ were tested for growth on oleate. The Mut$^-$Out$^-$ (methanol and oleate non-utilizing mutant) strains were then subjected to EM examination. All but one of the strains that fit this phenotypic profile were identified as *per* mutants. CAT was found to be mislocalized to the cytosol in both methanol- and oleate-induced cultures of the mutants. In contrast, AOX was almost absent from these mutants. It was suggested that AOX is synthesized but degraded rapidly. In contrast to *pas* and *per* mutants of *S. cerevisiae* and *H. polymorpha*, all the mutants of *P. pastoris* appear to harbor unusually small vesicular structures that may be peroxisomal ghosts.

Y. lipolytica. Recently, peroxisome assembly mutants (*pay* mutants) were reported in *Y. lipolytica* (Nuttley et al., 1993). These mutants were initially identified as oleic acid-non-utilizers and further characterized as peroxisome-deficient by immunofluorescence using antibodies against a SKL-containing peptide. Two *pay*

mutants were isolated that belonged to different complementation groups. In both mutants, CAT and β-hydroxyacyl-CoA dehydrogenase (a peroxisomal β-oxidation pathway enzyme) appeared to be cytosolic.

1.4.2.3 Isolation and characterization of peroxisomal genes

Mammalian peroxisomal genes. Two genes required for peroxisome biogenesis in mammals have been isolated. The first gene, *PAF-1* (peroxisome assembly factor-1), was cloned from a rat liver cDNA library by functional complementation of a peroxisome-deficient CHO cell line (Tsukamoto et al., 1991). It encodes a novel 35-kD protein that restores the assembly of peroxisomes and complements the defect in peroxisomal function of the mutant CHO cell line. The *PAF-1* product appears to be an integral membrane protein exposed on the cytosolic side of the peroxisome and is thought to be a component of the import machinery. The human homologue of *PAF-1* was cloned by screening a human liver cDNA library using the rat *PAF-1* cDNA as a probe (Shimozawa et al., 1992b). The two gene products were 88% identical. Both genes were able to rescue peroxisome biogenesis and peroxisomal biochemical function in fibroblasts from one complementation group of Zellweger syndrome cell line. The origin of the cell line was shown to be a patient who was homozygous for a point mutation that resulted in the premature termination of *PAF-1*.

The gene for PMP70, one of the major peroxisomal integral membrane proteins, was cloned from a rat liver cDNA expression library using anti-PMP70 polyclonal antibodies as a probe (Kamijo et al., 1990). The protein is markedly induced by peroxisomal proliferators along with peroxisomes and β-oxidation enzymes. The carboxyl-terminal sequence of PMP70 shows strong sequence similarity to a group of ATP-binding proteins (ATP-binding cassette transporter family) that are involved in diverse biological processes including active membrane transport of small and large

molecules (Higgins et al., 1986). PMP70 appears to be anchored in the peroxisomal membrane by its N-terminal domain with the C-terminus exposed to the cytosol and is hypothesized to be involved in transport of molecules through the peroxisomal membrane. The human homologue of PMP70 has also been cloned (Gartner et al., 1992; Kamijo et al., 1992). The nucleotide sequences of the two genes are 90.5% identical and the amino acid sequences of the two gene products are 95% identical. PMP70 gene is mutated in at least two complementation group I Zellweger patients, demonstrating that PMP70 is essential for peroxisome biogenesis.

Yeast peroxisomal genes. A number of genes whose products are required for peroxisome biogenesis have been cloned by functional complementation from yeast species, including *S. cerevisiae* (*PAS1, PAS2, PAS3, PAS8* and *PAS10*), *P. pastoris* (*PAS5* and *PAS8*) and *Y. lipolytica* (*PAY4*). In all cases, peroxisome biogenesis and missing peroxisomal functions were rescued by introducing the appropriate gene.

PAS1 encodes a 117-kD polypeptide (Pas1p) with two putative ATP binding domains that have similarity to a new family of putative ATPases (AAA family) involved in diverse biological processes including membrane fusion (Sec18p/NSF), cell cycle control (Cdc48p/VCP) and transcriptional regulation (TBP-1) (Erdmann et al., 1991). Pas1p appears to be rather hydrophilic, but its intracellular location is not yet known.

Pas2p, the *PAS2* gene product, has been characterized as a 21-kD protein related to the ubiquitin-conjugating (UBC) family of proteins which transfer ubiquitin to protein substrates as part of the ubiquitin conjugation cascade (Wiebel & Kunau, 1992). In *S. cerevisiae*, the ubiquitin pathway is known to be involved in a number of different cellular processes, e.g., protein degradation, cell cycle control and the secretory pathway (Jentsch, 1992; Johnson et al., 1992). Pas2p appears to be associated with peroxisomes, but its role in peroxisome biogenesis is not clear.

Pas3p is a 51-kD integral peroxisomal membrane protein with the bulk of the protein (C-terminus) exposed to the cytosol (Hohfeld et al., 1991). Pas3p showed no similarity to any other proteins.

The *S. cerevisiae PAS8* product, Pas8p, is a large polypeptide (116 kD) that contains two putative ATP-binding domains of AAA family type (Voorn-Brouwer et al., 1993). Similarity between Pas1p and Pas8p exists within the ATP-binding domain, but is restricted to this domain. Homologues of the *S. cerevisiae* Pas8p have been identified from *P. pastoris* (Pas5p, 127 kD) and *Y. lipolytica* (Pay4p, 112 kD) (Spong & Subramani, 1993; Nuttley et al., 1994). Pas5p appears to be associated with peroxisomes. It is interesting that *Y. lipolytica pay4* and *S. cerevisiae pas8* mutants contain no peroxisome (Per$^-$ phenotype), while the *P. pastoris pas5* mutant contains aberrant peroxisomal structures resembling membrane ghosts and appears to import a small amount of matrix enzymes into the ghosts, a phenomena also observed in some *H. polymorpha per* mutants.

Both *P. pastoris PAS8* (encoding a 65-kD protein) and its probable homologue, the *S. cerevisiae PAS10* (encoding a 69-kD protein), have been cloned (McCollum et al., 1993; van der Leij et al., 1993). The gene products appear to be novel members of the tetratricopeptide repeat gene (TPR) family that includes MAS70 (*S. cerevisiae*) and MOM72 (*Neurospora crassa*), which are receptors for mitochondrial protein import (Goebl & Yanagida, 1991). Mutants of both yeast species appear to be defective for growth but not for the division of peroxisomes, and they exhibit a specific defects in import of proteins with PTS1 but not PTS2. This observation is similar to that with several Zellweger syndrome cell lines. In *P. pastoris*, Pas8p was shown to associate with the peroxisomal membrane. *In vitro* translated *PAS8* protein binds specifically to a PTS1-containing peptide, suggesting it may be a PTS1 receptor or a factor that specifically interacts with a PTS1 receptor.

1.5 *H. polymorpha* as a Model System

The ability to combine genetics and biochemistry with yeast has been demonstrated to be a powerful tool to study diverse cell processes, including cell cycle, cellular signaling and protein secretion (Hartwell et al., 1974; Barr et al., 1989; Hartwell & Weinert, 1989). For peroxisome studies, *S. cerevisiae* was first chosen for molecular genetic studies owing to the considerable body of information accumulated on this organism. Growth of *S. cerevisiae* on oleic acid results in a significant proliferation of peroxisomes as well as induction of peroxisomal enzymes of the β-oxidation pathway (Veenhuis et al., 1987). *pas* mutants were first isolated in this organism and a number of *PAS* genes have been cloned (Erdmann et al., 1989; Kunau & Harting, 1992). Yet, *S. cerevisiae* grows poorly on oleic acid with growth ceasing after one or two generations, and peroxisomes in this organism still occupy only a relatively small proportion of total cytoplasmic volume after induction (Veenhuis, et al., 1987). This makes it difficult to perform many types of genetic experiments that require a determination of growth on oleate, to isolate peroxisomes for use in biochemical experiments and to locate proteins involved in peroxisome biogenesis in biochemical and EM studies.

H. polymorpha, a methylotrophic yeast, offers an attractive model system for these studies (Veenhuis, 1992). In this organism, detailed biochemical and physiological information exists in the metabolism of certain substrates, including methanol and ethanol as carbon sources, and D-alanine, urate acid and certain primary amines as nitrogen sources (Veenhuis & Harder, 1987). Growth on methanol results in the induction of peroxisomal methanol pathway enzymes including AOX, CAT and DHAS, and peroxisome growth and proliferation are massive (up to 80% of the total cell volume). Thus, *per* mutants are relatively easy to isolate (Cregg et al., 1990). In addition, *H. polymorpha* has a predominantly haploid life cycle and methods for classical- and

molecular-genetic manipulation of the organism are well developed (Gleeson & Subery, 1988; Faber et al., 1992). Although other methylotrophic yeasts including *P. pastoris* and *C. boidinii* have been used for peroxisome biogenesis studies, *H. polymorpha* has several advantages. *per* mutant cells from *H. polymorpha* uniquely contain a large AOX crystalloid that is readily visible under light microscope (van der Klei et al., 1991). Thus, *per* mutants can be efficiently identified within Mut$^-$ collections by light microscopy instead of tedious electron microscopic examination. Another unusual but useful characteristic of *H. polymorpha* is its relative thermotolerance. The yeast grows well at temperatures up to 45°C, which is particularly advantageous for isolating temperature-sensitive *per* mutants. With such mutants, it is possible to observe the biogenesis of new peroxisomes by shifting cells from nonpermissive to permissive temperature and to investigate interactions of gene products (Sulter et al., 1993b; Titorenko et al., 1993; Waterham et al., 1993).

1.6 Thesis Summary

This thesis describes the identification and characterization of *H. polymorpha* peroxisome-deficient mutants (*per* mutants) as well as the cloning and analysis of *PER8*, a gene required for peroxisome biogenesis in *H. polymorpha*. Dr. James M. Cregg initiated this program by isolating two *H. polymorpha per* mutants, demonstrating the possibility of utilizing *H. polymorpha* for genetic studies on peroxisomes.

Chapter 2 describes the identification and characterization of peroxisome-less (Per$^-$) mutants. Mut$^-$ mutants were subjected to electron microscopic examination and 38 of them were found to be completely devoid of peroxisomes. Complementation analysis and genetic mapping demonstrated that the strains represented 11 different *PER* genes. Two strains harbored dominant-negative alleles. The remaining 36 strains contained

recessive alleles. The expression and subcellular localization of peroxisomal matrix enzymes in these mutants were characterized by differential centrifugation and immunocytochemical methods. AOX and CAT, peroxisomal marker enzymes, were demonstrated to be mislocalized to the cytosol in all mutants.

The cloning and analysis of the *PER8* gene is described in Chapter 3. The gene was cloned by functional complementation of the *per8* mutant described in Chapter 3 and was localized to a 1.6-kb fragment by subcloning, northern blot analysis and the DNA sequencing of the gene. DNA sequence analysis revealed an open reading frame of 885 bp that encoded a 295 amino acid polypeptide with a calculated molecular mass of 34 kD. A mutant strain, in which a large portion of the *PER8* gene was deleted, was constructed and used to genetically demonstrate that the cloned gene was the same gene defective in the original *per8* mutant. The *PER8* gene product (Per8p) was expressed in *Escherichia coli* and used as antigen to raise antibodies. Biochemical and immunocytological experiments demonstrated that Per8p was a peroxisomal integral membrane protein and that it was concentrated in the membranes of newly formed organelles. Overexpression of *PER8* resulted in a markedly enhanced increase in peroxisome numbers. Thus, Per8p appears to play a role in peroxisome proliferation.

CHAPTER 2

CHARACTERIZATION OF PEROXISOME-DEFICIENT MUTANTS OF THE YEAST *HANSENULA POLYMORPHA*

2.1 Summary

In the methylotrophic yeast *Hansenula polymorpha*, approximately 25% of all methanol-utilization-defective (Mut$^-$) mutants are affected in genes required for peroxisome biogenesis (*PER* genes). Previously, we reported that one group of *per* mutants, termed Pim$^-$, are characterized by the presence of a few small peroxisomes with the bulk of peroxisomal enzymes located in the cytosol (Waterham, et al., 1992). Here, we describe a second major group of *per* mutants that were observed to be devoid of any peroxisome-like structure (Per$^-$). In each Per$^-$ mutant, the peroxisomal methanol-pathway enzymes alcohol oxidase, catalase and dihydroxyacetone synthase were present and active but located in the cytosol. Together, the Pim$^-$ and Per$^-$ mutant collections represented mutations in 14 different *PER* genes. Two of the genes, *PER5* and *PER7*, were represented by both dominant-negative and recessive alleles. Diploids resulting from crosses of dominant *per* strains and wild-type *H. polymorpha* were Mut$^-$ and harbored peroxisomes with abnormal morphology. This is the first report of dominant-negative mutations affecting peroxisome biogenesis.

2.2 Introduction

Eukaryotic cells are elaborately subdivided into functionally distinct membrane-bound compartments or organelles. Peroxisomes represent a unique class of such organelles present in virtually all eukaryotes (Lazarow and Fujiki, 1985; Borst, 1989; Subramani, 1993). Peroxisomal matrix enzymes are involved in a number of important metabolic processes but vary depending on the organism, tissue and environmental conditions. In humans, peroxisomes play an essential role in intermediary cellular metabolism as demonstrated by the existence of a family of lethal genetic disorders called Zellweger syndrome in which peroxisomes are defective (Lazarow and Moser, 1989).

It is now generally accepted that peroxisomes form by budding or dividing from pre-existing peroxisomes (Veenhuis et al., 1978). Peroxisomal proteins are encoded by nuclear genes, synthesized on free ribosomes, and imported post-translationally into the organelles in an ATP-dependent manner (Fujiki et al., 1984; Imanaka et al., 1987; Wendland and Subramani, 1993). Many peroxisomal matrix proteins are imported via a tripeptide targeting signal (SKL or conservative variants) located at the C-terminus (Gould et al., 1987). This peroxisomal targeting signal (PTS) is both necessary and sufficient to direct proteins into the organelle, and is evolutionarily conserved (Gould et al., 1989, 1990; Keller et al., 1991). Recent studies have revealed the existence of a second PTS which is present at the N-terminus of a few peroxisomal proteins (Osumi et al., 1991; Swinkels et al., 1991). Additional PTS systems may exist as well (de Hoop and AB, 1992). Aside from PTSs, general features of the molecular import machinery have yet to be elucidated.

Genetics has only recently been applied as an experimental tool to investigate peroxisomes (Lazarow, 1993). In fact, the first identified mutants affected in peroxisome function were human Zellweger patients (Goldfischer et al., 1973). Cells from such

patients are deficient in peroxisomes and as a result most peroxisomal enzymes are mislocalized to the cytosol (Lazarow and Moser, 1989). Evidence suggests that the deficiency is a consequence of defects in a major matrix protein import mechanism (Santos et al., 1988; Walton et al., 1992). Somatic cell fusion studies indicate that mutations in at least eight different genes are responsible for the disorder (Brul et al., 1988; Roscher et al., 1989). In addition to humans, peroxisome-deficient mutants have also been reported in Chinese hamster ovary cell lines (Zoeller and Raetz, 1986; Tsukamoto et al., 1990) and four yeast species: *Saccharomyces cerevisiae* (Erdmann et al., 1989; Kunau and Hartig, 1992), *Hansenula polymorpha* (Cregg et al., 1990; Didion and Roggenkamp, 1990; Waterham et al., 1992; Titorenko, et al., 1993), *Pichia pastoris* (Gould et al., 1992; Liu et al., 1992), and *Yarrowia lipolytica* (Nuttley et al., 1993).

The methylotrophic yeast *H. polymorpha* is an attractive model system for genetic studies on peroxisome biogenesis (Veenhuis, 1992). In this organism, detailed biochemical and physiological information exists on the role of peroxisomes in the metabolism of certain substrates including methanol and ethanol as carbon sources, and primary amines, D-alanine and uric acid as nitrogen sources (Veenhuis and Harder, 1987, 1991). In addition, methods for classical- and molecular-genetic manipulation of the organism are well developed (Cregg, 1987; Gleeson and Sudbery, 1988; Faber et al., 1992; Titorenko et al., 1993).

Previously, we described the isolation of two peroxisome-deficient mutants, termed *per* mutants, from a collection of strains defective in ability to grow on methanol (Mut^-) (Cregg et al., 1990). These mutants were identified by direct electron microscopic observation of methanol-induced cells. Continued screening of the Mut^- collection has resulted in the identification of an additional 58 *per* mutants. Based on peroxisome appearance in methanol-induced mutant cells, three distinct phenotypic classes could be distinguished. In the first class, peroxisomes are completely absent (Per^- phenotype). In

the second class, peroxisomes are present but abnormally small in size (Pim$^-$ phenotype). Recent studies on the Pim$^-$ mutants revealed that the bulk of peroxisomal enzymes are mislocalized to the cytosol in these mutants (Waterham et al., 1992). The third phenotypic class of *per* mutants induces peroxisomes that are normal in size and number but harbor an aberrant crystalline matrix substructure (Pss$^-$ phenotype) (Titorenko et al., 1993). In this report, we describe the identification and characterization of the Per$^-$ collection.

2.3 Materials and Methods

2.3.1 Strains, media and growth conditions

H. polymorpha strains used in this study are listed in Table 2.1. Cells were grown in either YPD medium (1% yeast extract, 2% peptone, 2% glucose) or in YNB minimal medium (0.67% yeast nitrogen base without amino acids) supplemented with 0.4% glucose. For growth on methanol, YNB minimal medium was prepared with 0.05% yeast extract and 0.5% methanol. Nutritional supplements for support of auxotrophic growth (methionine, leucine, adenine, arginine, or uracil and uridine) were added to 50 μg/ml as required. Sporulation (mating) medium was composed of 2% malt extract. Methanol-induced cells were prepared by preculturing strains twice in YPD medium for approximately 24 hours each, then shifting cultures to methanol by addition of one part of the second YPD culture to three parts of YNB medium with 0.5% methanol. All growth was at 37°C except where indicated in the text.

2.3.2 Genetic analysis

Genetic procedures such as mutagenesis, *per* mutant screening, mating, random spore analysis and backcrossing were performed as previously described (Gleeson and Sudbery, 1988). The standard procedure for complementation testing was modified to

effect a separation of mating and complementation phases. Each *per* mutant was first crossed with each of two different auxotrophically marked strains (usually *leu1* and *ade11* mutants but occasionally *met6, ura3* or *arg2*), and two sets of marked *per* spore products were isolated. One set of marked *per* strains was patched onto YPD agar plates (approximately 12 patches per plate) and the other marked set was spread as a lawn onto a series of YPD plates. The plates were then incubated overnight at 37°C. Next, cells from paired sets of patched plates and lawn plates were transferred by the replica-plating technique onto plates containing sporulation medium to initiate mating. After incubation at 37°C for 1 to 2 days, cells were further transferred to YNB minimal glucose plates to allow for the selective growth of prototrophic diploid cells and to prevent the growth of non-mated or self-mated cells. After incubation for 3 days at 37°C, the resulting prototrophic diploid colonies were spread on YNB minimal methanol plates to test for Mut phenotype.

Genetic mapping experiments were performed as described by Waterham et al. (1992).

2.3.3 Cellular fractionation

Mut^+ strains were precultured in YPD medium to an OD_{600} of approximately 0.75 and then shifted by centrifugation into one liter of YNB methanol medium to yield a starting OD_{600} of 0.005. Mut^- strains were precultured in one liter of YPD medium and the entire culture was shifted at an OD_{600} of approximately 0.75 into one liter of methanol medium. Cultures were incubated for 16 hours and harvested by centrifugation. Cells were washed three times with 10 ml of distilled water and resuspended in 4 ml of digestion buffer [5 mM K 3-(N-morpholino) propanesulfonate (pH 7.2), 0.5 M KCl, 10 mM Na_2SO_3] per gram of cells. Cells were then converted to protoplasts by the addition of Zymolyase 100T (ICN, Costa Mesa, CA) to 0.25 mg/ml and incubated at 37°C for 30

to 40 min with occasional shaking. All subsequent steps were carried out at 4°C. Protoplasts were diluted with 10 volumes of ice-cold 1 M sorbitol and centrifuged at 4,000 x g for 10 min. The resulting pellets were gently resuspended in sorbitol-MES buffer [1 M sorbitol, 5 mM K 2-(N-morpholino)ethanesulfonic acid, pH 5.5] at 1 ml per gram of cells. An equal volume of 0.25 M sorbitol, 5 mM MES (pH 5.5) was then added to the suspension while gently shaking and the mixtures were placed on ice for 10 min. An equal volume of 1.75 M sorbitol, 5 mM MES (pH 5.5) was then added sufficient to bring the solution back to 1 M sorbitol and samples were centrifuged at 1,000 x g for 20 min to remove unbroken cells and other debris. The supernatants were subjected to centrifugation at 25,000 x g for 25 min and the resulting pellets were resuspended in 100 ml of sorbitol-MES buffer and, along with supernatant fractions, were examined for enzyme activities.

2.3.4 Other methods

Electron microscopy and immunocytochemistry were performed as described previously (Waterham et al., 1992). Immunofluorescence experiments were done using rabbit polyclonal antibodies against total peroxisomal integral membrane proteins of *H. polymorpha* and fluorescein isothiocyanate (FITC) labeled anti rabbit antibodies (Sulter et al., 1993a). Total protein in samples was measured by the method of Bradford with bovine serum albumin as a standard (Bradford, 1976). AOX (van der Klei et al., 1990), catalase (Ueda et al., 1990), and fumarase (Tolbert, 1974) activities were measured according to published procedures.

2.4 Results

2.4.1 Identification of peroxisome-deficient mutants

In a previous report, we established that mutants with defects in peroxisome biogenesis exist as a subset within collections of Mut$^-$ mutants of *H. polymorpha* (Cregg et al., 1990). Based on this observation, we screened a collection of 260 Mut$^-$ strains by electron microscopy (EM) for mutants with defects in peroxisome morphology. Cells of each strain were induced in methanol medium and prepared for EM analysis. Under these conditions, ultra-thin sections from wild-type *H. polymorpha* and most Mut$^-$ strains showed virtually normal peroxisome proliferation (Figure 2.1A). However, 60 of the mutant strains had peroxisomal abnormalities (*per* mutants). The majority (38) of these were devoid of any recognizable peroxisome structures (Figure 2.1B) and this phenotypic class of *per* mutants was named Per$^-$. As indicated in the Introduction, two additional *per* mutant peroxisome phenotypes, Pim$^-$ and Pss$^-$, were also observed (reviewed in Veenhuis, 1992).

2.4.2 Genetic analyses

Each of the 38 Per$^-$ strains was crossed with one or more auxotrophically marked Mut$^+$ strains and diploids were selected on minimal methanol medium. Crosses involving 36 of the mutants yielded prototrophic Mut$^+$ diploid strains with normal-appearing peroxisomes (Per$^+$). (Analysis of the remaining two *per* mutants is described in the last section of the Results.) The resulting diploids were sporulated and random analysis of spore populations revealed a 1:1 ratio of Mut$^-$ and Mut$^+$ phenotypes from each. Selected spore products were further examined for peroxisomes and in each case peroxisome deficiency co-segregated with Mut$^-$ and vice versa. Thus, each of the 36 mutants appeared to be the consequence of a single recessive nuclear mutation.

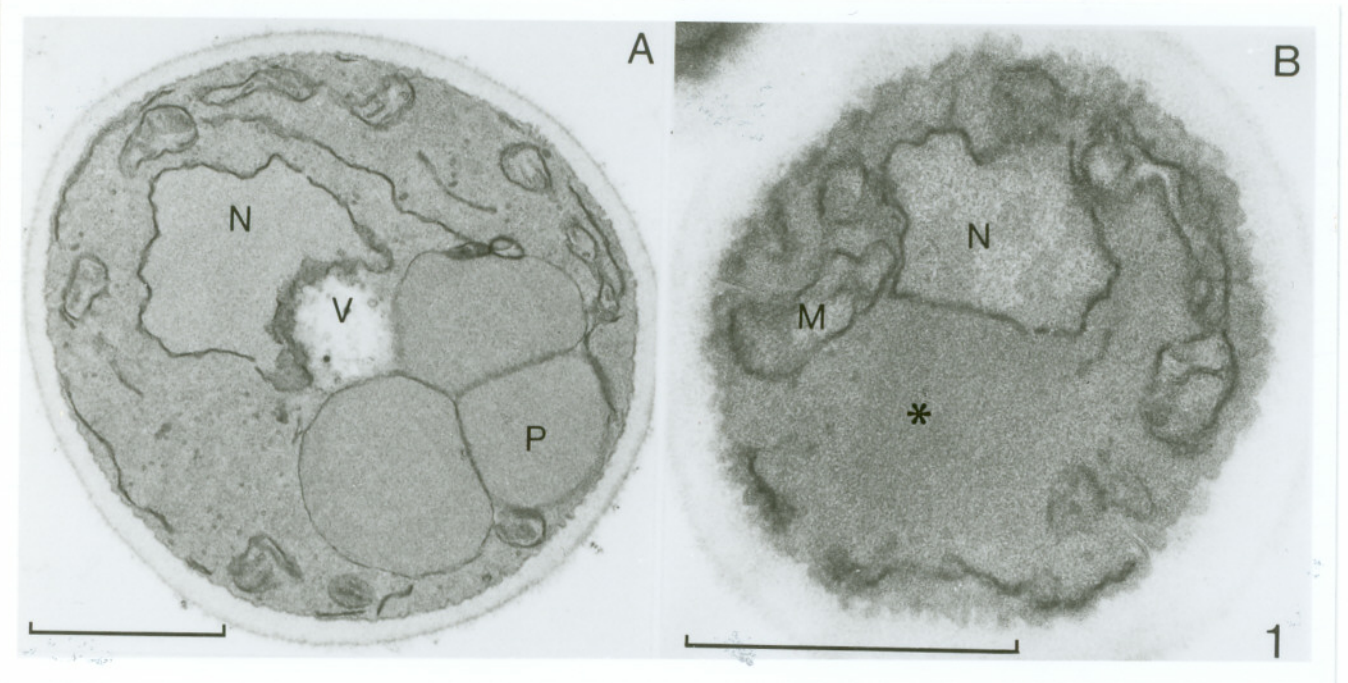


Figure 2.1. Ultrathin sections of a methanol-induced Per^+ (Mut^-) strain and a Per^- strain (*per2*) of *H. polymorpha*. Normal peroxisomes are observed in the Per^+ cell but are absent in the Per^- cell. Instead the cell contains a cytoplasmic AOX crystalloid. The structure of the crystalloid is poorly preserved due to $KMnO_4$-fixation. Abbreviations: M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. Bar, 1 mm.

The 36 *per* strains were each subjected to complementation analysis. To aid in the performance of this analysis, two sets of oppositely marked auxotrophic derivatives of each *per* strain were first constructed. The two sets of *per* strains were then crossed against each other and prototrophic diploids selected on minimal glucose medium. Diploid colonies were then tested for complementation of *per* alleles by their ability to grow on methanol. Results indicated that the 36 strains represented approximately 12 different complementation groups.

The assignment of mutant alleles to specific genes from the results of complementation testing is occasionally in error due to interallelic complementation between mutant alleles in the same gene or to unlinked noncomplementation between mutant alleles of different genes (Titorenko et al., 1993). To definitively assign *per* alleles to their proper *PER* genes, selected backcrossed *per* mutants were utilized in genetic mapping experiments. For this, each mutant was crossed with each of the other mutants and recombination distances were estimated by the random spore method. Eleven *PER* genes were defined by genetic mapping, one less than our original estimate of 12 due to one case of interallelic complementation between two mutant alleles.

We also crossed a mutant representative of each of the 11 *PER* genes with a mutant representative in each of the 5 Pim$^-$ groups defined as defective in genes *PER1* through *PER5* and the Pss$^-$ strain in *PER6*. The complementation and mapping results demonstrated that three representatives were alleles of previously defined *PER* genes (*PER1*, *PER3* and *PER5*), while the remaining 8 were defective in new *PER* genes which we named *PER7* through *PER14*. Thus, the total *per* mutant collection contained mutations in 14 different *PER* genes (see Table 2.1). As diagrammed in Figure 2.2, the mapping results revealed three linkage groups, each of which contained several *PER* genes. The relative order of genes in each group was determined through a series of three-factor crosses. Linkage group 1 included the following genes in order: *MET6*, *PER5*,

Table 2.1. *Hansenula polymorpha* peroxisome-deficient strains

Strain	Genotype	No. of Alleles*	Reference
CBS4732	wild type		CBS4732
A16	*leu1*		Veale et al. (1992)
MH122-3	*ura3*		Gleeson and Sudbery (1988)
MH120-1	*met6*		Gleeson and Sudbery (1988)
MH119-1	*ade11*		Gleeson and Sudbery (1988)
MH100-2	*arg2*		Gleeson and Sudbery (1988)
C36	*per1*	1 (11)	This study
C27	*per2*	1 (4)	Cregg et al. (1990)
C26	*per3*	3 (7)	This study
C16	*per4*	1 (4)	Waterham et al. (1992)
C79	*per5*	2	Waterham et al. (1992)
C4	$PER5^D$	1	This study
C72	*per6*	1 (2)	Titorenko et al. (1993)
C111	*per7*	1 (10)	This study
C35	$PER7^D$	1	This study
C76	*per8*	3	This study
B16	*per9*	2	This study
B64	*per10*	2	This study
C60	*per11*	1	This study
3P	*per12*	1	This study
C139	*per13*	2	This study
C88	*per14*	1	This study
C4/wt	$PER5^D$/*PER5*		This study
C35/wt	$PER7^D$/*PER7*		This study

*Number of allelic strains used in this study. Parentheses indicate the total number of *per* alleles including those previously described (Cregg et al., 1990; Waterham et al., 1992; Titorenko et al., 1993).

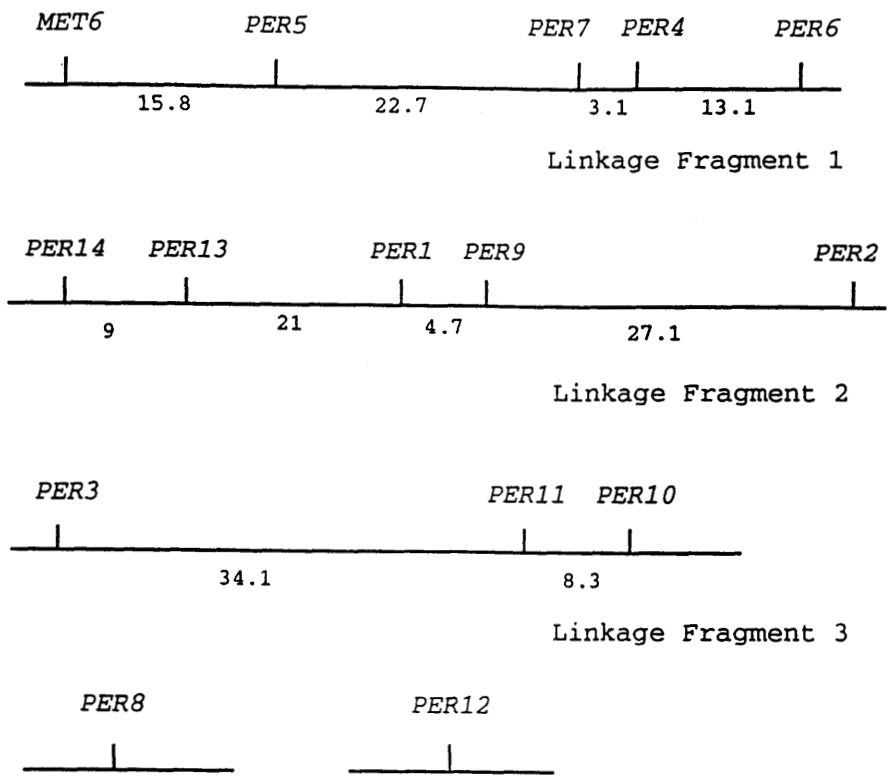


Figure 2.2. Genetic map of *H. polymorpha PER* genes as deduced by random spore analysis. A representative *per* mutant from each *PER* group was crossed with mutant representatives from each of the other groups and the resulting diploids were sporulated. Approximately 1000 spore products from each cross were germinated under non-selective growth conditions and tested for Mut phenotype. Crosses in which less than 25% of spore products were Mut^+ were examined further for potential genetic linkage by backcrossing Mut^- spore products to their respective parental *per* strains. Recombination distances are reported as the number of nonparental spore products (wild type + *per* double mutants) divided by the total number of spore products (nonparental + parental) expressed as a percentage. *PER8* and *PER12* are not linked to each other nor to any of the other *PER* genes.

PER7, PER4 and *PER6*. (*MET6* is a locus previously identified by Gleeson and Sudbery, 1988). Linkage group 2 contained the following genes: *PER14*, *PER13*, *PER1*, *PER9* and *PER2*. Linkage group 3 contained genes: *PER3*, *PER10* and *PER11*. Two genes, *PER8* and *PER12*, were unlinked to each other or to any of the other *PER* genes.

Methanol-induced cells of backcrossed mutant representatives in each *PER* gene were serially sectioned and carefully examined for peroxisomes by EM. Most mutants were still Per$^-$ including: C36 (*per1*), C79 (*per5*), C111 (*per7*), C76 (*per8*), B16 (*per9*), B64 (*per10*), C60 (*per11*), and 3P (*per12*). However, two strains, C26 (*per3*) and C139 (*per13*) that were Per$^-$ prior to backcrossing were Pim$^-$ after backcrossing, and another, C88 (*per14*), was Pss$^-$. Thus, it appeared that, prior to backcrossing, these three strains contained secondary mutations that influenced the mutant peroxisome phenotype. Interestingly, two *PER* genes, *PER1*, and *PER5* were represented by both Per$^-$ and Pim$^-$ mutant alleles. Thus, it appears that, for these two genes at least, the mutant peroxisome phenotype was allele specific.

2.4.3 Localization of peroxisomal enzymes

Backcrossed mutant representatives in each newly-identified *PER* gene (*PER7-PER14*) and newly-identified Per$^-$ representatives in *PER1* and *PER5* were examined for the presence, activity and subcellular location of selected peroxisomal enzymes. In methanol-induced cells of each *per* mutant, substantial activity for both alcohol oxidase (AOX) and catalase
(CAT) activity was present (Figure 2.3A). Homogenized protoplasts prepared from these cultures were then subjected to differential centrifugation and the sedimentation pattern of AOX and CAT determined (Figure 2.3B). In wild-type preparations, approximately 60% of AOX and 40% of CAT activity was present in the organelle pellet. A significant amount of activity was also present in the supernatant due to organelle breakage during the

A. Peroxisomal Enzyme Activities

	wt	1	3	5	7	8	9	10	11	12	13	14
CAT	100	83	103	163	79	121	190	81	26	133	43	211
AOX	100	59	134	122	66	34	72	25	24	100	42	218

B. Sedimentation Patterns of Peroxisomal Enzymes

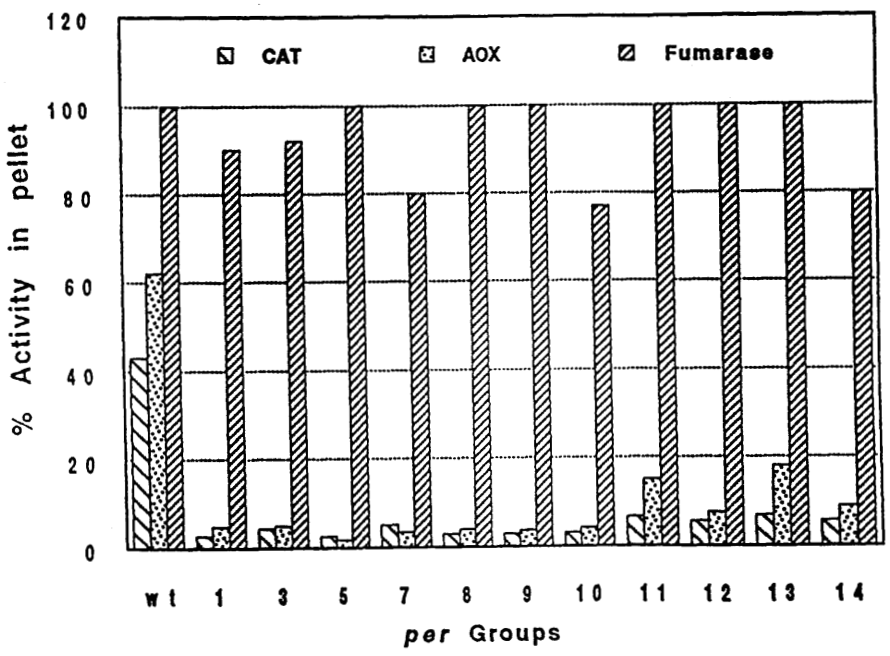


Figure 2.3. Activity and location of peroxisomal enzymes in methanol-induced per mutant cells. (A) Percentage of AOX and CAT activities relative to that in wild-type cells. (B) Percentage of AOX, CAT and fumarase activities in post-25,000 x g organelle pellet after differential centrifugation of homogenized protoplasts.

procedure. However, with each of the *per* mutant preparations, most AOX and CAT activity was found in the supernatant, indicating that the enzymes were located in the cytosol. These results were not a consequence of unequal handling of *per* cells during preparation since fumarase, a mitochondrial marker enzyme, remained primarily in the pellet.

The cytosolic location of AOX and CAT was confirmed by immunocytochemical experiments. In methanol-induced Per$^-$ cells, AOX typically formed a large cytosolic crystalloid (Figure 2.4A) that was readily observed by light microscopy (inset to Figure 2.4B). In contrast, CAT appeared to be randomly dispersed throughout the cytosol in each mutant (not shown). Dihydroxyacetone synthase (DHAS), the third peroxisomal methanol-pathway enzyme, was also present and located primarily within the crystalloid in each mutant (Figure 2.4B).

2.4.4 Identification and analysis of dominant-negative mutants

For two Per$^-$ strains, C4 and C35, we were unable to introduce an auxotrophic marker by mating and therefore, could not perform complementation testing initially. Their apparent failure to mate may have been the result of the presence of secondary mutations affecting this process or of dominant-negative (*PER^D*) alleles which would prevent the growth and selection of diploids on minimal methanol medium. (A critical requirement of mating homothallic yeasts such as *H. polymorpha* is the ability to selectively grow crossed diploid lines in the presence of self-mated diploids and non-mated parental cells.) To further investigate these mutants, spontaneous uracil-requiring (Ura$^-$) derivatives of C4 and C35 were isolated by selection for resistance to 5-fluoro-orotic acid (Boeke et al., 1987). The Ura$^-$ derivatives were then mated with auxotrophically marked strains and observed to readily form prototrophic diploids, a result that suggested the presence of a *PER^D* allele in C4 and C35. The dominant-negative

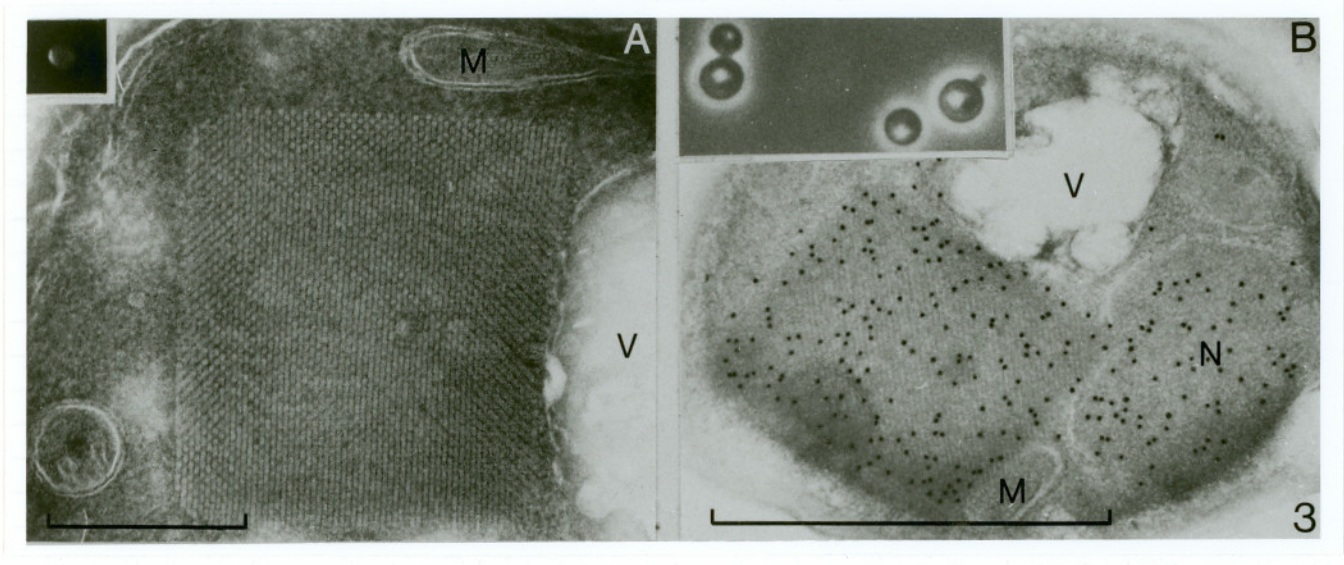


Figure 2.4. Micrographs of a methanol-induced Per⁻ strain (C111, *per7*). (A) EM detail of an ultrathin cryosection of the strain demonstrating the cytosolic AOX crystalloid. (A, inset) Characteristic immunofluorescent pattern observed after incubation of the strain with antibodies specific for total PMPs and FITC. (B) Accumulation of DHAS protein in AOX crystalloids revealed through immunocytochemical staining using specific antibodies against DHAS and protein A gold. (B, inset) Phase contrast light microscopy of AOX crystalloids in induced Per⁻ cells. Bar, 1 mm. Light micrographs are at 3,000 x.

nature of the alleles was confirmed by the observation that C4/wt and C35/wt diploid strains were Mut$^-$. Further testing revealed that C35/wt diploids were actually cold sensitive (Mut$^-$ at 25 and 30°C but Mut$^+$ at 37 and 43°C), whereas C4/wt strains were Mut$^-$ at all temperatures tested (25 to 43°C).

Peroxisome morphology in methanol-induced cultures of diploid C4/wt and C35/wt strains was examined by EM. Control diploid strains constructed of crosses between strains with recessive *per* alleles and either wild type or complementing *per* allele pairs contained normal peroxisomes (Figure 2.5A). In contrast, C4/wt diploids contained only a few small peroxisomes along with a large cytosolic AOX crystalloid (Figure 2.5B), a phenotype similar to that seen in haploid Pim$^-$ mutants of *H. polymorpha* (Waterham et al., 1992). C35/wt diploids which were cold-sensitive for methanol growth were also cold-sensitive with respect to peroxisome development with normal-appearing organelles at 43°C (Figure 2.5C). At 25°C, C35/wt diploids contained a few large peroxisomes. Furthermore, the large mature organelles frequently displayed distinctive discontinuities in their membranes (Figure 2.5D). It is difficult to understand how such membrane holes could exist *in vivo*. However, since they were not observed in wild-type control samples, they could not be entirely due to preparation artifacts. Perhaps the peroxisomal membrane is weak at one or more points and the stress of sample preparation then disrupts the membrane at these locations.

The *PER*^{D} alleles were assigned to *PER* gene groups by first crossing Ura$^-$ derivatives of C4 and C35 to Leu$^-$-marked recessive *per* representatives of each group and selecting for diploids on minimal glucose medium. As expected, all of the resulting diploids were Mut$^-$. Diploid strains derived from each cross were then sporulated and approximately 1,000 spore products from each cross were examined for Mut phenotype. Approximately 25% of the spore products were Mut$^+$ from all but two crosses. The two exceptions were C4 crossed with the *PER5* representative, and C35 crossed with the

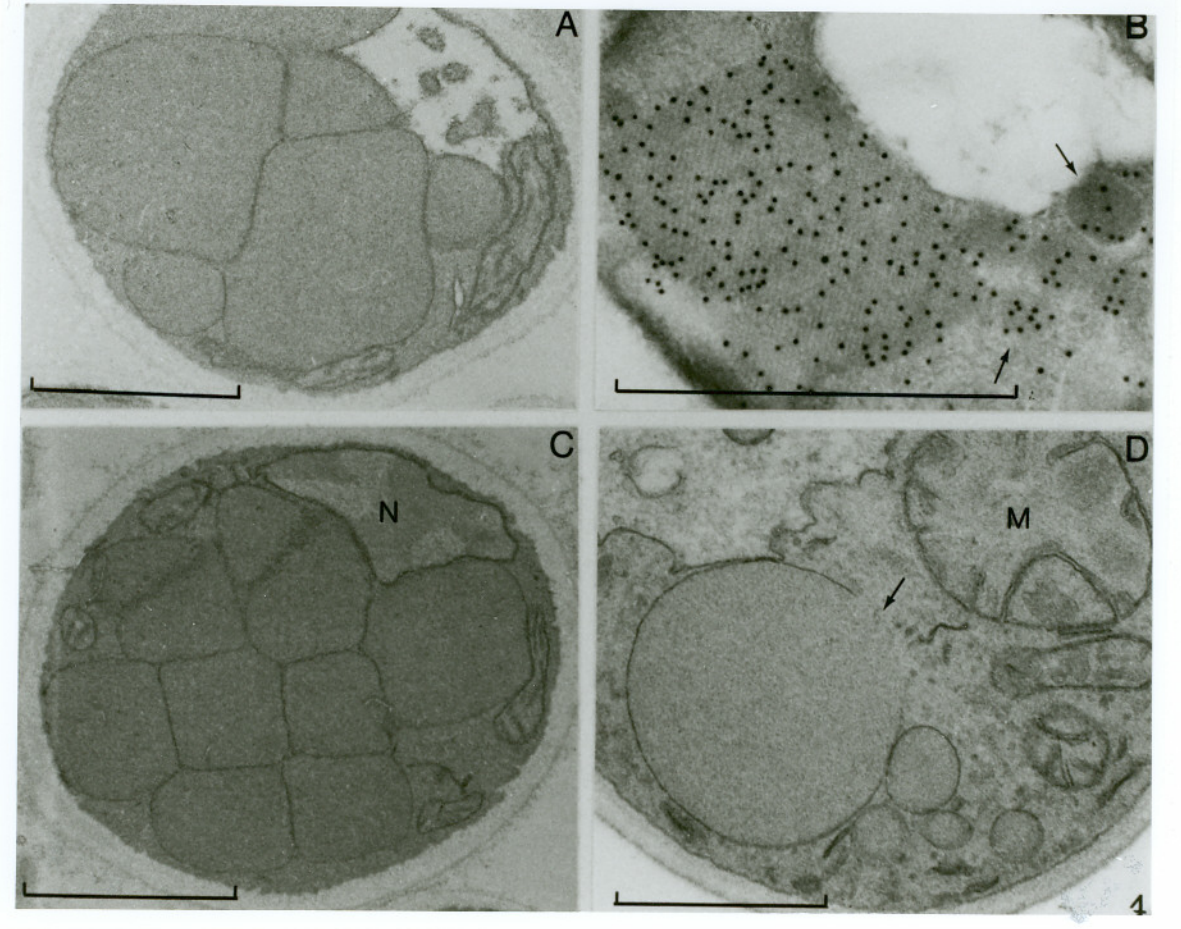


Figure 2.5. Electron micrographs of selected methanol-induced *H. polymorpha* diploid strains. (A) Normal peroxisomes in a diploid constructed from two recessive Per$^-$ alleles *per2* and *per7*. (B) C4/wt (*PER5*^{D}/*PER5*) diploid cell showing only a few small peroxisomes in conjunction with a single large cytosolic AOX crystalloid (Lowicryl, anti-AOX antibodies, protein A/gold). (C) C35/wt (*PER7*^{D}/*PER7*) diploid cell cultured in methanol at 43^{o}C showing normal peroxisome proliferation and morphology. (D) The same strain in C cultured in methanol at 25^{o}C. Note the interruption in the membrane of the mature peroxisome (arrow). Bar, 1 mm.

PER7 representative, which both yielded no Mut$^+$ spores. The absence of Mut$^+$ spore products demonstrated tight linkage between alleles and indicated that the mutations in C4 and C35 were *PER*^{D} alleles of *PER5* and *PER7*, respectively.

2.5 Discussion

In previous reports, we established that functional peroxisomes are essential to support growth of *H. polymorpha* on methanol as sole carbon and energy source, and that mutants with defects in the organelle (*per* mutants) can be found among collections of Mut$^-$ mutants of this yeast (Cregg et al., 1990; van der Klei et al., 1991a). We have exploited this discovery by isolating and characterizing 60 *per* mutants. The mutants can be differentiated into three phenotypic classes based on peroxisome morphology in methanol-induced mutant cells. Two mutants contain peroxisomes in which the normally ordered structure of the crystalline matrix, primarily composed of AOX, is disorganized (Titorenko et al., 1993). This phenotype is named Pss$^-$ for peroxisomes with abnormal matrix substructure. The two Pss$^-$ mutant alleles are in *PER3* and *PER6*. Another 20 *per* strains contain only very small peroxisomes (Waterham et al., 1992). In methanol-induced cells of these mutants, peroxisomal matrix enzymes are present and active. However, only a fraction is imported into the small organelles. This phenotype is named Pim$^-$ for peroxisome import defective. We previously described Pim$^-$ alleles in 5 genes, *PER1* through *PER5*.

In this report, we describe the identification and characterization of the third phenotypic class of *per* mutants which we call Per$^-$. Methanol-induced cells of each Per$^-$ mutant were carefully searched by EM for peroxisome-like structures but none could be found. Thus, either Per$^-$ mutants do not contain peroxisomes or they are unrecognizable. In Per$^-$ mutants, the peroxisomal enzymes AOX, CAT and DHAS are present but

mislocalized to the cytosol. As also seen in Pim$^-$ mutants, AOX forms into a single active non-membrane-bounded crystalloid structure (van der Klei et al., 1991b). One Per$^-$ mutant, C111 (a *PER7* representative), has been examined for the fate of peroxisomal membrane proteins (PMPs) (Sulter et al., 1993b). In this mutant, PMPs exist as part of a cytosolic proteinaceous phospholipid aggregate in each cell. The aggregate, which is thought to be a possible peroxisomal remnant, can be observed by immunofluorescence microscopy as a punctate structure within C111 cells (inset to Figure 2.4A). Similar punctate structures are observed in each Per$^-$ mutant, suggesting that PMP-containing aggregates are a common feature of this class of *per* mutant.

Two of the Per$^-$ mutants harbor dominant-negative mutant (*PER^D*) alleles. Diploid strains constructed between either of these mutants and wild-type *H. polymorpha* are Mut. One, C35 (*PER7^D*), forms heterozygous diploids that display a cold-sensitive Mut phenotype. The other, C4 (*PER5^D*), forms diploids that are Mut$^-$ at all temperatures. Consistent with their Mut$^-$ phenotype, peroxisome morphology in methanol-induced diploid cells is also abnormal, although in both cases the morphology is different from that of the haploid parents. Whereas both C4 and C35 are Per$^-$, the *PER5^D*/wt diploid contains small peroxisomes along with a large cytosolic AOX crystalloid, while the *PER7^D*/wt diploid contains peroxisomes with membrane disruptions at 25°C and normal organelles at 37°C. Both *PER^D* alleles are in genes for which numerous recessive *per* alleles also exist and their isolation suggests that the affected *PER* products take part in important protein-protein interactions either with themselves as homomultimers or with other peroxisomal proteins. These are the first dominant mutations affecting peroxisome biogenesis to be reported and the first dominant negative mutants to be described in a methylotrophic yeast species. Furthermore, only a few reports of dominant negative mutants affecting organelle biogenesis exist in the literature (Robinson et al., 1988; Vater et al., 1992).

It is tempting to speculate on the nature of the gene products whose defects could lead to a Per$^-$ phenotype, i.e., the complete absence of the organelles. However, few clues are available for meaningful conjecture. The mutants could be defective in the peroxisome fission process, in segregation of the organelles to daughter cells, in a protein recognition/import system or in regulatory factors involved in expression of other products required for peroxisome biogenesis. The Per$^-$ mutants are most likely not defective in factors required for catabolite derepression such as the *SNF* products as are several of the *S. cerevisiae* peroxisome-deficient (*pas*) mutants (Simon et al., 1992; van der Leij et al., 1992), since all our *per* mutants grow normally on other derepression-requiring carbon sources including ethanol and glycerol, and induce relatively normal levels of methanol pathway enzymes.

We would also like to understand the functional significance, if any, of the Per$^-$, Pim$^-$ and Pss$^-$ phenotypic classes. Evidence suggests that in certain cases, Per$^-$ is the phenotype of a totally defective *PER* gene while Pim$^-$ is the result of a partial or "leaky" mutation. First, several instances have been found in which different mutant alleles in the same *PER* gene result in strains with different peroxisome phenotypes (Titorenko et al., 1992; this report). As examples, mutant alleles of *PER1* and *PER5* can be either Per$^-$ or Pim$^-$ while *PER3* mutant alleles are either Pim$^-$ or Pss$^-$. Second, two *per* mutants (*per3* and *per13*) which were initially identified as Per$^-$ became Pim$^-$ after backcrossing. Third, a temperature-sensitive *per* mutant that is Per$^-$ at non-permissive temperature displays a Pim$^-$ phenotype when cultured at an intermediate temperature (Waterham et al., 1993). However, it is also possible that some *PER* genes are involved in more than one function in peroxisome biogenesis and yield mutant alleles that are specifically defective in each function. A definitive answer for each *PER* gene must await the construction of null alleles from the cloned genes.

Among yeast species, *H. polymorpha* has several unique advantages as a model system for molecular genetic studies on peroxisomes (Veenhuis, 1992). Due to its requirement for peroxisomes for growth on methanol and the large size of the organelles in methanol-induced cells, *per* mutants are relatively easy to isolate. The task of identifying *per* mutants is made even easier by the observation that methanol-induced *per* mutant cells (Per$^-$ and Pim$^-$ classes) uniquely contain a large AOX crystalloid that is readily visible under the light microscope (van der Klei et al., 1991b). Thus, *per* mutants can be efficiently identified within Mut$^-$ collections of *H. polymorpha* by light microscopy while EM observation can be reserved for confirmation of a peroxisome-deficient diagnosis. Another unusual but useful characteristic of *H. polymorpha* is its relative thermo-tolerance. The yeast grows well at temperatures of up to 45°C, which is particularly advantageous for isolating temperature-sensitive *per* mutants. With temperature-sensitive *per* alleles it is possible to observe the biogenesis of new peroxisomes by shifting cultures from nonpermissive to permissive temperature or to watch the effects of the withdrawal of a required peroxisomal product by shifting from permissive to nonpermissive temperature (Sulter et al., 1993b; Waterham et al., 1993).

To date, we have identified mutant alleles in 14 different *PER* genes. The existence of this large collection of *H. polymorpha per* mutants along with efficient transformation vectors and host strains for this yeast have allowed us to take the next major step in our studies, the cloning of *H. polymorpha PER* genes by functional complementation. Several *PER* genes have been isolated to date (Waterham et al., 1994; unpublished data). The predicted amino acid sequences of *PER* products along with detailed biochemical information on the effect of their malfunction on the cell should provide useful insights into the function of specific *PER* products and their roles in peroxisome biogenesis.

CHAPTER 3

THE *HANSENULA POLYMORPHA PER8* GENE ENCODES A NOVEL PEROXISOMAL INTEGRAL MEMBRANE PROTEIN INVOLVED IN PROLIFERATION

3.1 Summary

We previously described the isolation of mutants of the methylotrophic yeast *Hansenula polymorpha* that are defective in peroxisome biogenesis. Here, we describe the characterization of one of these mutants, *per8*, and the cloning of the *PER8* gene. In either methanol or methylamine medium, conditions that normally induce the organelles, *per8* cells contain no peroxisome-like structures and peroxisomal enzymes are located in the cytosol. The sequence of *PER8* predicts that its product (Per8p) is a novel polypeptide of 34 kD, and antibodies against Per8p recognize a protein of 31 kD. Analysis of the primary sequence of Per8p revealed a 39 amino acid cysteine-rich segment with similarity to the C3HC4 family of zinc-finger motifs. Overexpression of *PER8* results in a markedly enhanced increase in peroxisome numbers. We show that Per8p is an integral membrane protein of the peroxisome and that it is concentrated in the membranes of newly formed organelles. We propose that Per8p is a component of the molecular machinery that controls the proliferation of this organelle.

3.2 Introduction

Eukaryotic cells are divided into a variety of membrane-bound compartments or organelles, each responsible for performing specific metabolic functions. To maintain each organelle, the cell must correctly direct specific sets of proteins to their proper subcellular locations and, as the cell grows and divides, the organelles must be duplicated. For some organelles, such as the endoplasmic reticulum, mitochondrion and peroxisome, the cell can also increase organelle numbers in response to certain environmental stimuli (Bolender et al., 1973; Attardi et al., 1988; Braunbeck et al., 1991; Dreyer et al., 1992; Luiken et al., 1992). Of these organelle biogenesis tasks, significant progress has been made in elucidating the molecular mechanisms responsible for protein sorting, whereas little is known regarding the mechanisms responsible for organelle duplication/proliferation.

We are interested in understanding biogenesis in peroxisomes (glyoxysomes, glycosomes), a class of single membrane-bound organelles that exists in virtually all cells and is the site of a number of important oxidative reactions (Borst, 1989;Veenhuis and Harder, 1991; van den Bosch, 1992; Subramani, 1993). An unusual characteristic of peroxisomes relative to other organelles is their functional diversity; that is, the specific metabolic pathways found in the organelle vary depending upon the organism, the tissue and its environment. The importance of peroxisomes to humans is dramatically demonstrated by a family of lethal genetic diseases called Zellweger syndrome in which peroxisomes appear to be absent from or deficient in patient cells (Lazarow and Moser, 1989). Thus, peroxisomes are clearly essential for human survival.

In recent years, basic features of peroxisome biogenesis have emerged. Proteins destined for the peroxisome are synthesized on free ribosomes, usually at their mature sizes, and post-translationally imported into the organelle (Roa and Blobel, 1983; Fujiki et

al., 1986). For import of peroxisomal matrix enzymes, two distinct peroxisomal targeting signals (PTSs) have been defined (for review see de Hoop and AB, 1992). The most common signal, PTS1, is a carboxy-terminal tripeptide that is typically SKL or a conservative variant (Gould et al., 1987; Gould et al., 1990a; Aitchison et al., 1991; Keller et al., 1991). The second, PTS2, is found on only a few peroxisomal enzymes such as mammalian and yeast 3-ketoacyl-CoA thiolases and yeast amine oxidase (Osumi et al., 1991; Swinkels et al., 1991; Glover et al., 1994; K.-N. Faber and M. Veenhuis, personal communication). PTS2 is located near the amino-terminus and is cleaved in some instances (rat thiolase) but not in others (yeast thiolase). Peroxisomal membrane proteins and some matrix enzymes do not appear to have either PTS; therefore, it is likely that one or more additional PTSs exist. Aside from targeting signals, import machinery components remain largely unidentified with the exception of the *Pichia pastoris PAS8* gene product which may be a receptor for proteins bearing PTS1 (McCollum et al., 1993).

Less is known regarding the cell's ability to control peroxisome volume and number. Like mitochondria, peroxisomes are thought to duplicate by fission from preexisting organelles and to actively migrate into daughter cells (Veenhuis et al., 1979; Attardi and Schatz, 1988). The number of peroxisomes per cell can increase dramatically in response to metabolic needs. For example, certain hypolipidemic drugs such as clofibrate induce peroxisomes to proliferate in rat liver cells (Lock et al., 1989). However, the most extreme example of a proliferative response is observed in methylotrophic yeasts such as *Hansenula polymorpha* where methanol induces a massive increase in peroxisome size and number (Veenhuis et al., 1979). In glucose-grown cells of this yeast, only one or a few small peroxisomes are present, whereas in methanol, peroxisomes can account for as much as 80% of total cell volume. Interestingly, when peroxisomal enzymes are expressed at high levels in glucose-grown cells, the organelles increase in size but not number (Godecke et al., 1989; Roggenkamp et al., 1989). Thus,

it appears that peroxisome size may be a reflection of the amount of matrix protein, while proliferation is controlled by a separate mechanism.

H. polymorpha is an attractive model system for genetic studies on peroxisome biogenesis (Veenhuis et al., 1992). In this organism, detailed physiological, biochemical and ultrastructural information exists on the role of peroxisomes in the metabolism of a variety of unusual carbon sources nitrogen sources (Veenhuis and Harder, 1987; Veenhuis and Harder, 1991). In addition, methods for classical- and molecular-genetic manipulation of the organism are well developed (Cregg, 1987; Gleeson and Sudbery, 1988; Faber et al., 1992; Titorenko et al., 1993). In previous reports, we have described the isolation and characterization of *H. polymorpha* mutants that are defective in peroxisome biogenesis (*per* mutants) (Cregg et al., 1990; Waterham et al., 1992; Titorenko et al., 1993). In this report, we describe the characterization of one mutant, *per8*, and the molecular cloning and characterization of the *PER8* gene and its product. We show that *PER8* encodes a 34-kD peroxisomal integral membrane protein that appears to play a role in peroxisome proliferation. To our knowledge, this is the first description of a component of an organelle's proliferation machinery.

3.3 Materials and Methods

3.3.1 Strains, media and microbial techniques

H. polymorpha strains used in this study are listed in Table 3.1. *H. polymorpha* cultures were grown at 37°C in a complex medium (YPD) composed of 1% yeast extract, 2% peptone and 2% dextrose, or in one of the following minimal media: YNB medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate) supplemented with either 0.4% dextrose or 0.5% methanol and 0.05% yeast extract; MAD medium (0.17% yeast nitrogen base without amino acids and ammonium

Table 3.1. *H. polymorpha* strains used in study of *PER8* gene

Strain	Genotype	Source or reference
CBS4732	wild type	CBS4732
A16	*leu1*	Veale et al., 1992
C76	*per8-1*	This study
CT100	*per8-1 leu1*	This study
CT101	*per8Δ::SLEU2*	This study
CT102	*per8Δ::SLEU2 ade11*	This study
CT103	*per8-1 leu1* P_{MOX}*-PER8*	This study
CT104	*per1 leu 1*	This study

sulfate, 0.25% methylamine, 0.25% dextrose). Nutritional supplements for growth of auxotrophic strains were added to 50 µg/ml as required. Sporulation (mating) medium was composed of 2% malt extract. Genetic manipulations of *H. polymorpha* have been described (Gleeson and Sudbery, 1988; Cregg et al., 1990; Faber et al., 1992). Yeast transformations were performed by the Klebe procedure (Klebe et al., 1983). Cultivation of *Escherichia coli* strains and recombinant DNA techniques were performed as described (Sambrook et al., 1989).

3.3.2 Cell fractionation

Methanol-utilizing (Mut^+) strains were precultured in YPD medium to an OD_{600} of approximately 0.75 and then shifted by centrifugation into one liter of YNB methanol medium at a starting OD_{600} of 0.005. Methanol-utilization-defective (Mut^-) strains were precultured in one liter of YPD medium and the entire culture was shifted at an OD_{600} of approximately 0.75 into one liter of methanol medium. Cultures were incubated for 16 hours and harvested by centrifugation. For growth on methylamine, cultures were precultured in YNB dextrose and shifted by centrifugation into MAD medium at a starting density of 0.1 OD_{600} units and harvested at an OD_{600} of approximately 1.0. Cells were washed three times with 10 ml of distilled water and resuspended in 4 ml of digestion buffer [5 mM K 3-(N-morpholino) propanesulfonate (pH 7.2), 0.5 M KCl, 10 mM Na_2SO_3] per gram of cells. Cells were then converted to protoplasts by the addition of Zymolyase 100T (ICN, Costa Mesa, CA) to 0.25 mg/ml and incubated at 37°C for 30 to 40 min with occasional shaking. All subsequent steps were carried out at 4°C. Protoplasts were diluted with 10 volumes of ice-cold 1 M sorbitol and centrifuged at 4,000 x g for 10 min. The resulting pellets were gently resuspended in sorbitol-MES buffer [1 M sorbitol, 5 mM K 2-(N-morpholino)ethanesulfonic acid, pH 5.5] at 1 ml per gram of cells. An equal volume of 0.25 M sorbitol, 5 mM MES (pH 5.5) was then added to the

suspension while gently shaking and the mixtures were placed on ice for 10 min. A volume of 1.75 M sorbitol, 5 mM MES (pH 5.5) was then added sufficient to bring the solution back to 1 M sorbitol and samples were centrifuged at 1,000 x g for 20 min to remove unbroken cells and other debris. The supernatants were subjected to centrifugation at 25,000 x g for 25 min and the resulting pellets were resuspended in 100 ml of sorbitol-MES buffer and, along with supernatant fractions, were examined for enzyme activities. For purification of peroxisomes, pellets resulting from the differential centrifugation procedure described above were layered over a discontinuous sucrose gradient, centrifuged, and fractions collected as described previously (Douma et al., 1985).

Extraction of peroxisomes with triethanolamine and carbonate were performed as described in Waterham et al. (1994). Triton X-114 extractions were done as previously described (Bordier, 1981). Urea extractions were performed as described (Cunningham et al., 1989) except that the 6 M urea solution was in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl.

3.3.3 Genomic DNA library construction

The *H. polymorpha* genomic DNA library used to clone *PER8* was constructed by inserting total DNA from wild-type *H. polymorpha* strain CBS4732 into the *H. polymorpha-E. coli* shuttle plasmid pYT3. This plasmid was constructed by inserting the *Saccharomyces cerevisiae LEU2* (*SLEU2*) gene on a 2.2-kb SalI-XhoI fragment into the SalI site of pUC19. *SLEU2* serves as a selectable marker for transformation into *leu1* strains of *H. polymorpha* (Gleeson et al., 1986). pYT3 also contains an *H. polymorpha* NruI-PvuII DNA fragment of 2.5 kb that contains autonomous replication sequence (HARS) activity in *H. polymorpha* inserted into the SmaI site of pUC19. To construct the library, total DNA from *H. polymorpha* was extracted and partially digested with Sau3AI.

Fragments of 5 to 10 kb were pooled and further size-selected by sucrose density gradient centrifugation. To receive the DNA fragments, pYT3 was digested with BamHI and treated with calf intestine alkaline phosphatase. Approximately equal molar amounts of fragmented DNA and vector were mixed and ligated with T4 DNA ligase. Ligation products were transformed into *E. coli* strain DH5a and approximately 29,000 colonies were collected. Plasmid DNA was extracted from these cells and purified as described (Sambrook et al., 1989). The library was stored as plasmid DNA in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA at -20°C.

3.3.4 Isolation and characterization of the *PER8* Gene

Cells of *H. polymorpha* strain CT100 (*per8-1 leu1*) were transformed with the *H. polymorpha* DNA library and Leu^+ colonies were selected on YNB glucose medium agar plates. Approximately 2,000 Leu^+ transformants were washed from the plates and inoculated into liquid YNB methanol medium. Samples of the liquid culture were periodically spread onto YNB methanol medium agar plates. After three days, growth on methanol was observed and the culture was harvested. Total DNA was extracted from these cells and plasmids were recovered by transforming the DNA into *E. coli*. Plasmid DNA was isolated from 10 *E. coli* transformants and all were found to contain pYT3 with an insert of 5.8 kb. One of these plasmids, pYT7 (shown in Figure 3.4) was retransformed into CT100 by selection for Leu^+. The resulting Leu^+ transformants were then replica plated on methanol medium agar plates and all were observed to also be Mut^+. To further define the location of *PER8* sequences, selected subfragments from pYT7 were subcloned into pYT3 and tested for Mut^+ complementation of CT100. An EcoRI-NruI fragment of 1.8 kb was identified and inserted into pBluescript II SK^+ (Stratagene, La Jolla, CA) in both orientations and a series of subclones containing deletions of the fragment were generated by limited digestion with exonuclease III (Henikoff, 1987). The

DNA sequence of both strands of the insert was then determined by the dideoxy chain-termination method (Sanger and Coulson, 1975). DNA and predicted protein sequences were analyzed using MacVector software (IBI, New Haven, CT) and compared with the GenBank data base.

3.3.5 Construction of *PER8* disruption strain

A vector designed to delete all but the first 68 amino acids of the predicted *PER8* product (Per8p) was constructed in five steps. In the first, pBluescript II SK^+ was digested with EcoRI and KpnI and an adaptor oligonucleotide (AATTAGTCGACGAATTCAGTACTGGTAC and CAGTACTGAATTCGTCGACT) was inserted. This step resulted in the removal of pBluescript restriction sites between EcoRI and KpnI, destruction of the pBluescript EcoRI site, and generation of the following new restriction sites: SalI, EcoRI, ScaI, KpnI. The resulting vector was named pDT2. In the second step, a 520-bp fragment composed of sequences flanking the 5' end of *PER8* plus sequences encoding the first 68 amino acids of the gene was amplified by the polymerase chain reaction (PCR) method from plasmid pYT7 using the 5' primer TTGGTACCAGTACTAGCCGCTAAAAAAACCGGGCGT and 3' primer GGGTCGACGAATTCGGAACCCACCAATGTTGTGAGA. The PCR product was digested with EcoRI and KpnI and inserted into EcoRI-KpnI-digested pDT2 to make pDT3. In the third step, a second fragment of 630-bp composed of sequences starting immediately 3' of the TAA translation stop codon of *PER8* was PCR amplified using the 5' primer AAAGGGAACAAAAGCTGGAGCT and 3' primer ATGGATCCTCTAGATTGTAAAACGACGGCCAGTGA. This 3' PCR product was digested with XbaI and inserted into XbaI-digested pDT3 to make pDT4. For the fourth step, additional 3' sequences from the *PER8* locus were added by digesting pDT4 with NruI and inserting a 1.7-kb NruI-EcoRV fragment to create pDT5. In the fifth step, a 2.2-

kb SalI-XhoI fragment encoding the *S. cerevisiae LEU2* gene (*SLEU2*) was inserted into SalI-digested pDT5 between the two *PER8*-flanking fragments to make plasmid pDT6. This plasmid was digested with ScaI and NruI to generate a 3.2-kb fragment containing the *PER8*-deletion allele (*per8Δ*) and terminating in sequences flanking *PER8* as shown in Figure 3.6A. The *per8Δ* fragment was transformed into *H. polymorpha* strain A16 (*leu1*) by selection for Leu^+ and transformants were screened for ones which were Mut^-. DNAs from Leu^+ and Mut^- transformants were then examined by the Southern hybridization method for proper targeting of the *per8Δ* fragment into the *PER8* locus as described in the results section and shown in Figure 3.6B.

3.3.6 Preparation of antibodies against the PER8 product

Per8p was expressed in *E. coli* as a fusion with the maltose binding protein (MAL) using a kit supplied by New England Biolabs (Beverly, MA). To construct the strain, the MAL expression vector, pMAL-c2, was first modified to accept a *PER8*-containing DNA fragment in the proper reading frame by digesting the vector with EcoRI and HindIII, and inserting adaptor oligonucleotides (AATTCAAGCTTGGATCCCCTTGG and AGCTGCAAGGGGATCCAAGCTTG) to make pFT2. Insertion of this adaptor resulted in the following changes in order: (1) restoration of the EcoRI site of pMAL-c2; (2) addition of a new HindIII site that was in frame with the HindIII site located near the 5' end of *PER8* coding sequences; (3) addition of new BamHI and StyI sites; and (4) destruction of the original HindIII site of pMAL-c2. A 1.1-kb HindIII-StyI fragment encoding Per8p amino acids 3 through 295 was isolated from pYT7 and inserted into HindIII- and StyI-digested pFT2. Proper in-frame fusion of *malE-PER8* sequences was confirmed by DNA sequencing one of the resulting plasmids, pFT4. This plasmid was then transformed into *E. coli* strain TB1. A transformant was selected and grown and MAL-Per8p fusion protein was induced by addition of 0.3 mM isopropylthiogalactoside.

After two hours at 37°C, cells were harvested, lysed by sonication and the fusion protein was purified by amylose affinity and DEAE-sepharose chromatography as recommended by the supplier.

Purified MAL-Per8p fusion protein was used to immunize rabbits (Josman Laboratories, San Jose, CA). The resulting antiserum was affinity purified by a described procedure (Raymond et al., 1990). Crude serum was loaded onto a MAL-Per8p fusion protein column and bound antibodies were eluted with 0.2 M glycine (pH 2.4). The resulting preparation was then passed through a column containing total *H. polymorpha* protein from the *per8Δ* strain CT101 and another column containing MAL. The resulting antibody preparation was concentrated using a Centriprep-10 concentrator (Amicon, Beverly, MA) and stored at -70°C in 1 x PBS buffer (Sambrook et al., 1989).

3.3.7 Construction of *PER8* overexpression strain

Strain CT103, which overexpresses *PER8* in methanol medium, was constructed by transformation of *per8-1 leu1* strain CT100 with plasmid pET4. This plasmid was created from YIp32, a plasmid composed of a 2.2-kb SalI-XhoI fragment encoding the *S. cerevisiae LEU2* gene inserted at the SalI site of pBR322. To construct pET4, the HindIII site of YIp32 was first destroyed by digesting the plasmid with HindIII, filling in the resulting termini with dNTPs using the Klenow fragment of DNA polymerase I and ligating to create YIp32-HK. Second, a 2.6-kb BamHI-BglII fragment that contains the *H. polymorpha* alcohol oxidase promoter (P_{MOX}) was inserted from plasmid pHIPX2 (Faber et al., 1992). This fragment was inserted into the BamHI site of YIP32-HK to make pET1. Third, pET1 was digested with HindIII and SmaI, and an adaptor oligonucleotide (AGCTGATGTTTAAGCTTTCGCGACCC and GGGTCGCGAAAGCTTAAACATC) was inserted to make pET3. The adaptor made the

following changes in order: (1) destruction of the HindIII site at the junction of the adaptor and pET3; (2) addition of sequences encoding the methionine initiator ATG and the second amino acid of Per8p; (3) addition of a new HindIII in frame with the 5' HindIII site of *PER8*; and (4) addition of an NruI site. Finally, a 1.3-kb HindIII-NruI fragment encoding all but the first two amino acids of the *PER8* gene product was inserted into HindIII- and SmaI-digested pET3. The resulting plasmid pET4 contains the *PER8* gene under methanol oxidase (MOX) promoter control and was digested with StuI prior to transformation into CT100 to direct the plasmid to integrate at the P_{MOX} locus. Proper integration of pET4 was confirmed by Southern blot analysis.

3.3.8 Miscellaneous methods

Total protein in samples was measured by the method of Bradford with bovine serum albumin as a standard (Bradford, 1976). AOX (van der Klei et al., 1990), catalase (Ueda et al., 1990), AMO (Zwart et al., 1980) and fumarase (Tolbert, 1974) activities were measured according to published procedures. Northern and Southern procedures were as described (Sambrook et al., 1989). Immunobloting was performed using either the ProtoBlot system of Promega (Madison, WI) or the ECL system of Amersham (Arlington Heights, IL) as directed by the suppliers. Electron microscopy and immunogold-electron microscopy with anti-Per8p rabbit antibodies were performed as described (Waterham et al., 1992).

3.4 Results

3.4.1 *per8* cells lack morphologically recognizable peroxisomes

H. polymorpha strain C76 (*per8-1*) was one of a number of mutants isolated by screening a collection of Mut$^-$ strains for ones in which intact peroxisomes were absent

(Cregg et al., 1990; Waterham et al., 1992; Titorenko et al., 1993). In methanol-grown wild-type *H. polymorpha*, peroxisomes are abundant (Figure 3.1A). In contrast, an exhaustive electron microscopic (EM) search of serially sectioned cells of the *per8-1* strain induced on methanol failed to reveal any peroxisome-like structures (Figure 3.1B). Thus, in *per8-1* cells, either peroxisomes were not present, or were morphologically unrecognizable, a phenotype we refer to as Per$^-$ (Titorenko et al., 1993). A striking feature of methanol-induced *per8-1* cells was the presence of a single large cytosolic crystalloid that was readily observed by phase contrast light microscopy as a bright yellow cubic structure tumbling within each cell (Figure 3.1B, inset). This crystalloid structure was primarily composed of alcohol oxidase (AOX) (Figure 3.1C) and is a common characteristic of most *H. polymorpha per* mutants examined to date (van der Klei et al., 1991).

Additional evidence for the absence of peroxisomes in *per8-1* was obtained from biochemical experiments designed to determine the location of peroxisomal enzymes. Enzyme activity measurements of extracts prepared from methanol-induced *per8-1* cells indicated that catalase (CAT) and AOX were present at substantial levels (Figure 3.2A). The third peroxisomal methanol-pathway enzyme, dihydroxyacetone synthase (DHAS), was also present as demonstrated by immunobloting using antibodies against DHAS (data not shown). The location of these enzymes was investigated by subcellular fractionation of homogenized *per8-1* spheroplasts. After low-speed centrifugation to remove remaining whole cells, protoplasts, and nuclei, small organelles (primarily peroxisomes and mitochondria) were sedimented by centrifugation at 25,000 x g. At this force, a major portion of the activity for peroxisomal enzymes in wild-type cells was present in the organelle pellet (Figure 3.2B). However, a significant amount of activity was also present in the supernatant due to enzyme leakage or organelle breakage or both. In contrast, after centrifugation of *per8-1* preparations, almost all AOX and CAT activity and DHAS protein

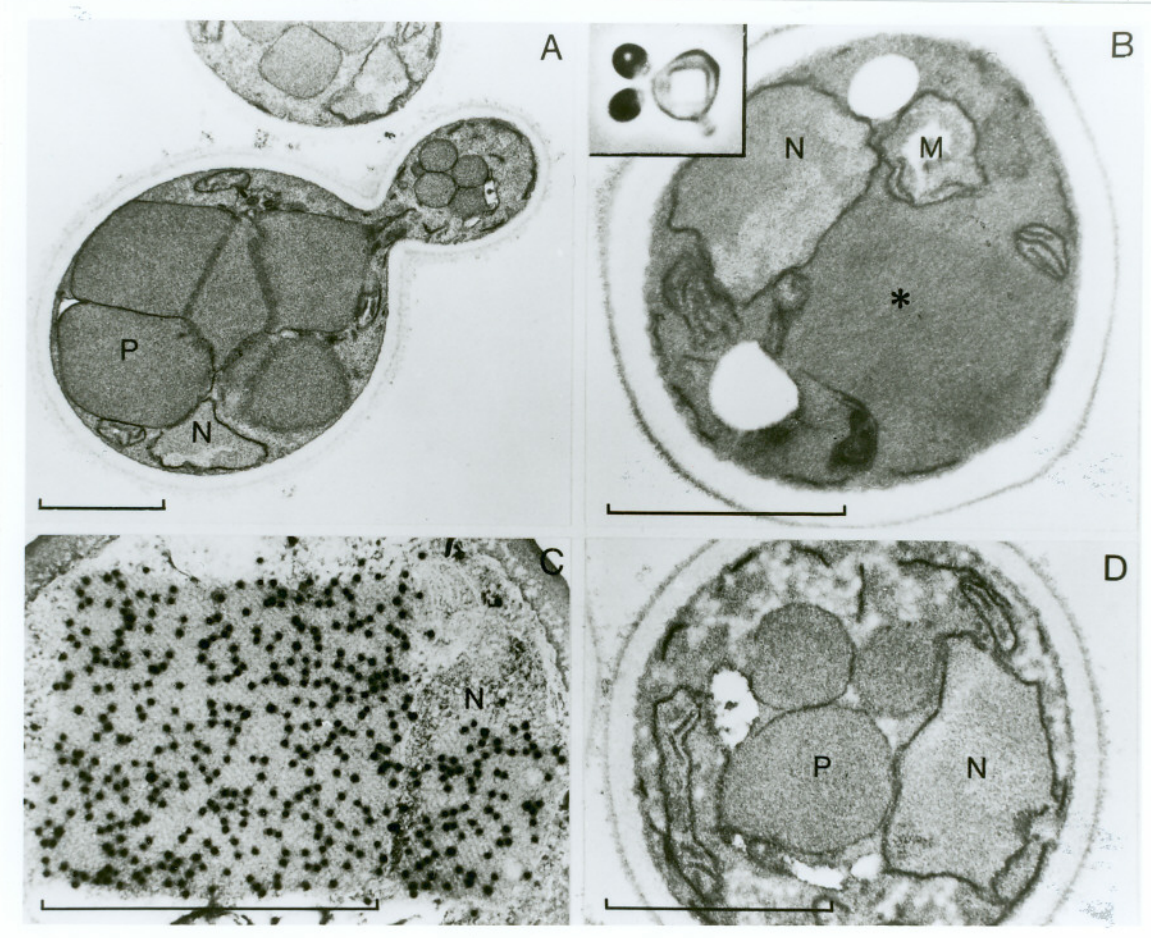


Figure 3.1. Electron micrographs showing subcellular morphology of selected *H. polymorpha* strains. (*A*) Methanol-grown wild-type cells contain numerous large peroxisomes (P); mitochondrion (M); nucleus (N). (*B*) Methanol-induced *per8-1* cells contain a large cytosolic AOX crystalloid (∗) but no peroxisomes; (inset *B*) phase-contrast light micrograph of *per8-1*. (*C*) Immuno-gold labeling of a methanol-induced *per8-1* cell with anti-AOX antibodies showing the presence of AOX in the cytosolic crystalloid. A second small crystalloid is also present in the nucleus of this cell, a common occurrence in *per8-1* and other *per* cells. (*D*) *per8-1* cells transformed with a complementing DNA fragment grow on methanol and contain normal peroxisomes. Bar, 1.0 mm.

A

	PER8	*per8-1*	*per8-1+PER8*	*per8Δ*
Methanol				
CAT	100	121	73	90
AOX	100	34	57	22
Methylamine				
CAT	100	368	120	399
AMO	100	253	108	233

B

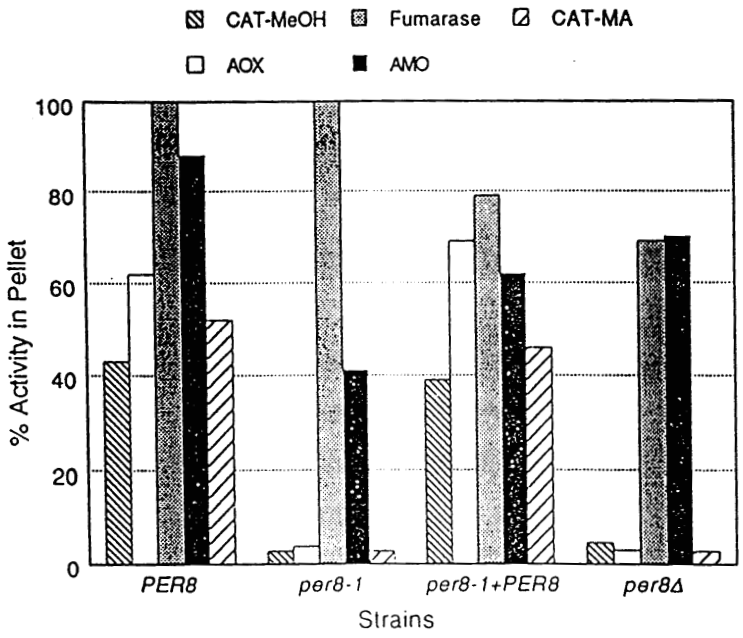
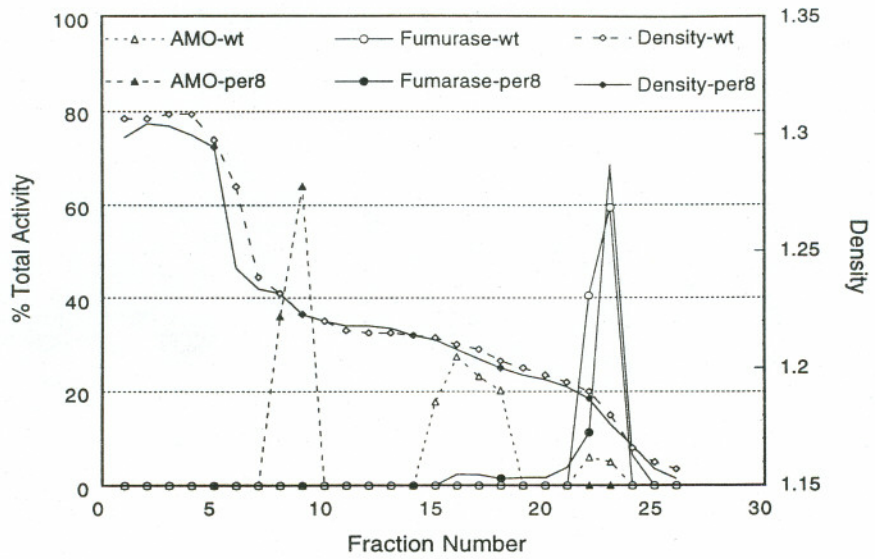


Figure 3.2. *per8* cells induce peroxisomal enzymes but do not import them into peroxisomes. (*A*) AOX and CAT_{MeOH} activities in methanol-induced strains and AMO and CAT_{MA} activities in methylamine-grown strains reported as a percentage of that in wild-type (*PER8*) cells. *per8-1* + *PER8* is strain CT100 (*per8-1 leu1*) transformed with *PER8*-containing plasmid pYT7. (*B*) Percentage of enzyme activity in post-25,000 x g organelle pellet after differential centrifugation of homogenized protoplasts.

were located in the supernatant, indicating that these enzymes were located in the cytosol (Figure 3.2B). These results could not be due to unequal handling of *per8* cells during the fractionation procedures since fumarase, a mitochondrial marker enzyme, remained primarily in the pellet.

In *H. polymorpha*, the utilization of methylamine as a nitrogen source requires the peroxisomal enzyme amine oxidase (AMO) (Zwart et al., 1980; Sulter et al., 1990). AMO has recently been shown to be directed to peroxisomes via a PTS2 sequence (Faber and Veenhuis, unpublished results), whereas AOX, CAT and DHAS each contain a PTS1 sequence (Didion and Roggenkamp, 1992; Hansen et al., 1992). Thus, the fate of AMO is an indicator of PTS2 system function. In methylamine-grown *per8-1* cells, AMO and CAT activity levels were greater than twice those in wild-type cells (Figure 3.2A). After differential centrifugation, AMO activity was present primarily in the pellet fraction while CAT was mostly in the supernatant fraction (Figure 3.2B). These results suggested that AMO may be in peroxisomes and therefore, that *per8-1* cells may be specifically defective in the import of PTS1-containing enzymes. On the other hand, AMO has been shown to form cytosolic aggregates in methylamine-grown cells of some *per* mutants (Sulter et al., 1990). Therefore, it was possible that in *per8-1* cells, AMO also formed aggregates and that these aggregates sedimented during differential centrifugation. To differentiate between these explanations, the pellet fractions resulting from differential centrifugation were subjected to sucrose density gradient centrifugation. As shown in Figure 3.3A, AMO from *per8-1* cells sedimented to a significantly higher density than peroxisome-enclosed AMO from wild-type cells, suggesting that, in *per8-1*, AMO was in a structure with little or no associated membrane such as a protein aggregate. Methylamine-grown *per8-1* cells were also examined by EM for peroxisomes. As with methanol-induced cells, no peroxisome-like structures could be found (data not shown). In addition, immunocytochemical results using antibodies against AMO showed that AMO was indeed

A



B

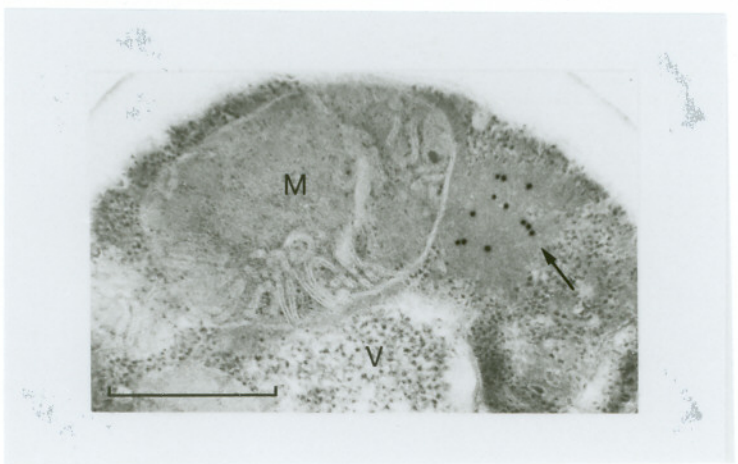


Figure 3.3. AMO is located primarily in protein aggregates in *per8-1* cells. (*A*) Sucrose density gradient profile of AMO activity in crude organelle pellet derived from methylamine-grown *per8-1* and wild-type cells. (B) Section of methylamine-grown *per8-1* cell after immuno-gold labeling with anti-AMO antibodies. Bar, 0.5 mm.

located primarily in aggregates (Figure 3.3B). We concluded that AMO, like the other peroxisomal enzymes, was not in peroxisomes and that the absence of peroxisomes in *per8-1* cells was not a methanol- or PTS1-specific defect but a general impairment in peroxisome biogenesis.

3.4.2 Isolation and characterization of the *PER8* gene

The *PER8* gene was isolated from an *H. polymorpha* genomic DNA library by functional complementation of *per8* strain CT100 (*per8-1 leu1*). Library transformants were initially selected for leucine prototrophy (Leu^+) on glucose medium and subsequently selected for growth on methanol (Mut^+). Total DNA was extracted from a pool of Leu^+ Mut^+ transformants and plasmids were recovered by transforming the DNA into *E. coli.* A plasmid, designated pYT7 (Figure 3.4), was isolated that co-transformed the *per8-1 leu1* strain to both Leu^+ and Mut^+ simultaneously. *per8-1* cells transformed with pYT7 contained normal-appearing peroxisomes (Figure 3.1D) and sedimentable activity for AOX, CAT and AMO (Figure 3.2B). Restriction mapping of pYT7 revealed an *H. polymorpha* DNA insert of 5.8 kb. By subcloning selected sub-fragments from pYT7, the complementing activity was located within a 1.6-kb EcoRI-NruI fragment. In northern blots, the 1.6-kb fragment hybridized to a single transcript of 1.4 kb that was present at low levels in glucose-grown wild-type *H. polymorpha* and induced approximately 5-fold in methanol-grown cells (Figure 3.5, lanes 4 and 5). Since methanol induces peroxisome proliferation, the higher level of this transcript in methanol-grown cells was consistent with it being the product of a peroxisomal gene.

The DNA sequence of the 1.6-kb fragment from pYT7 revealed a single long open reading frame (ORF) of 885 bp with the potential of encoding a

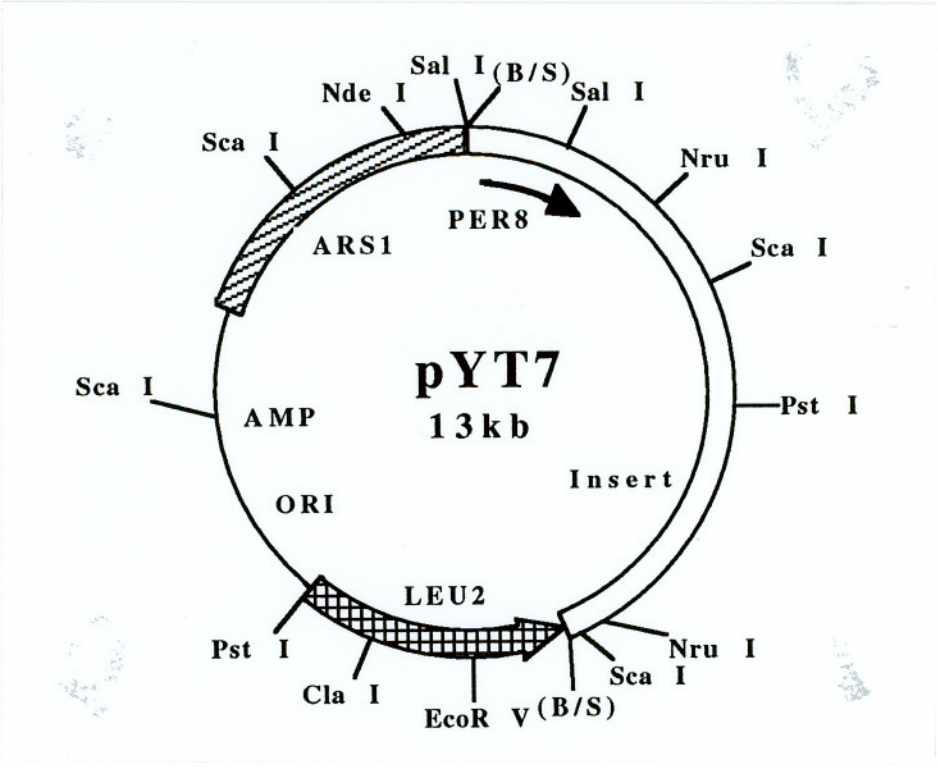


Figure 3.4. Restriction endonuclease cleavage site map of *PER8*-containing plasmid pYT7.

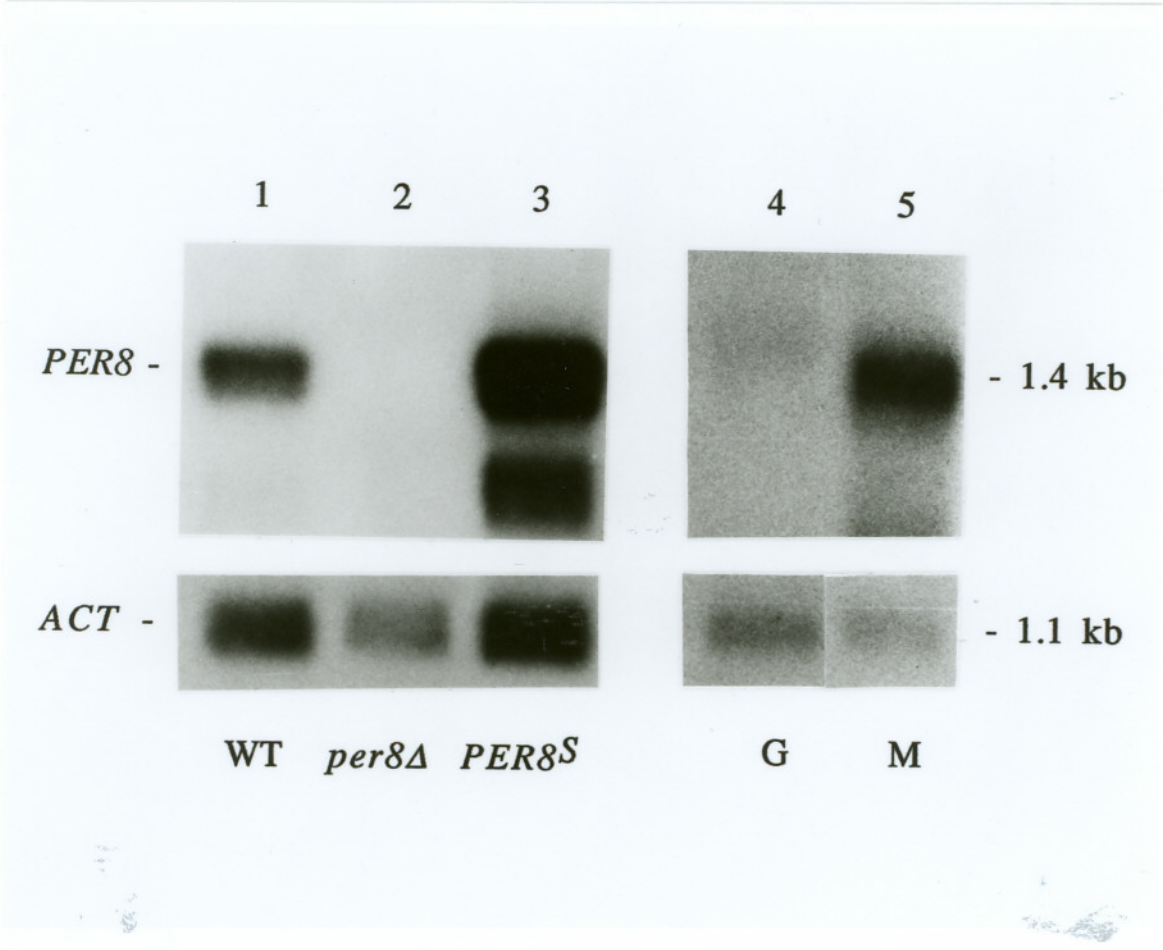


Figure 3.5. Northern blot showing induction of *PER8* message levels by methanol. All lanes contain 6 µg of total RNA. Methanol-grown wild-type cells contain a 1.4-kb *PER8* message (lane 1) that is not present in methanol-induced *per8Δ* cells (lane 2) but is present at a high level in methanol-grown cells of a strain that expresses *PER8* under control of P_{MOX} (lane 3). (lanes 4 and 5) *PER8* message level in glucose- and methanol-grown wild-type *H. polymorpha* cells, respectively. As a control, filters were hybridized with a labeled DNA fragment encoding the *S. cerevisiae* actin gene (*ACT*). *ACT* message levels in methanol-grown cells are approximately three-fold lower than in glucose-grown cells.

polypeptide of 295 amino acids (~34 kD) (Figure 3.6A). Several results indicated that this ORF encoded the *PER8* gene product. First, as described in the next section, an *H. polymorpha* strain in which most of the ORF was deleted had the same phenotype as the *per8-1* strain and was an allele of *per8-1*. Second, antibodies prepared against the product of the ORF specifically reacted with a protein of 31 kD (Figure 3.11A), a mass that was in approximate agreement with the mass predicted from the ORF. Translation of Per8p most likely begins at the assigned ATG since initiation at the next ATG in the ORF was predicted to generate a protein of only ~19 kD. However, since the apparent mass was less than the predicted mass, it was conceivable that Per8p may be post-translationally processed. Third, as described below, the product of the ORF is a peroxisomal protein. Hydropathy analysis indicated that Per8p was of average overall hydrophobicity with no apparent α-helical transmembrane domains (Figure 3.6B). Database searches revealed no other proteins with overall sequence similarity to Per8p. The only identifiable feature of the predicted Per8p sequence was a segment of 39 amino acids near its carboxy-terminus that was a perfect match to the C3HC4 motif ($\underline{C}X_2\underline{C}X_{11\text{-}30}\underline{C}X\underline{H}X_2\underline{C}X_2\underline{C}X_{10\text{-}18}\underline{C}PX\underline{C}$), a subgroup within the cysteine-rich zinc-finger domain family (Figure 3.6A and C) (Freemont et al., 1991). Outside the C3HC4 region, the Per8p sequence displayed no further similarity to any of these proteins.

3.4.3 A *PER8*-deleted strain is peroxisome-deficient

An *H. polymorpha* strain in which most of *PER8* was deleted was created by the gene replacement method (Rothstein, 1983). For the replacement, a plasmid was constructed in which 700 bp of *PER8* coding sequence (nucleotides 206 to 906 encoding amino acids 69 to 295 in Figure 3.6A) was removed and replaced with a

A

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-324                                                                    atcatcat
-316  caagccgctaaaaaaaccgggcgtgccttcgtctccggtagtggaagagactcccagccgagcagaaacgcccacgggg
-237  agcaaggcgcgggcaagtggtctttcgggtgagcttgatcgctcaggaagaccgtgcaagcggtggaccaaaaagacac
-158  gtgtgttcaagactttcagcggctgggacgtcgaggttgggatctggagaccgtgtacgtagggttttgcttttgttct
 -79  atgtacgatttcactaaaaatatcaattacgaatgggctaatatttgcgtctggctaggctaaataattattccttttt

   1  ATG TTT AAG CTT TTG TCT TTT GCC AAT GCT CCA GCG ATT GTG CGG GCC AAC CAG AAG GAT
   1   M   F   K   L   L   S   F   A   N   A   P   A   I   V   R   A   N   Q   K   D

  61  TCA TAT TTT GAG TCG AGG CTG CAC AAC CAG CTC TTA GAC GTT GTG AAG GCC ATC AAA GGC
  21   S   Y   F   E   S   R   L   H   N   Q   L   L   D   V   V   K   A   I   K   G

 121  TCT CAC TTT GTC CAC AAG TAC CCG GAG GAG CTG CGA ACC CTG GCC ACG GCT CTT TAT CTG
  41   S   H   F   V   H   K   Y   P   E   E   L   R   T   L   A   T   A   L   Y   L

 181  TGT CTC ACA ACA TTG GTG GGT TCC AAG ACG CTG GGC GAG GAG TAC GTG GAT CTG GTG TAT
  61   C   L   T   T   L   V   G   S   K   T   L   G   E   E   Y   V   D   L   V   Y

 241  GTG AGT CGT GAC GGA CGC AAG ATA CCG AAA TTT GCG TCC CGA TTT GGC TTT GTC GTG GCG
  81   V   S   R   D   G   R   K   I   P   K   F   A   S   R   F   G   F   V   V   A

 301  TAT GTG CTC TTC CCC TAT GCT GTT CGC CAA CTG CTT CAA AAA CTG AAA GCA CAG CAA AGC
 101   Y   V   L   F   P   Y   A   V   R   Q   L   L   Q   K   L   K   A   Q   Q   S

 361  CGG CTA GCA CAA CTG GTT TCC GGA GTC AGC TAC ATG AAC GTG ATG GAT CTT TTG AAT CTC
 121   R   L   A   Q   L   V   S   G   V   S   Y   M   N   V   M   D   L   L   N   L

 421  CAT CTG GCG CTC TTC TAC TTC ACG GGC AAA TAC TAT CAG TTT GCG AAA AGA CTC TTT GGT
 141   H   L   A   L   F   Y   F   T   G   K   Y   Y   Q   F   A   K   R   L   F   G

 481  CTG CGA TAT GCG TTT GGC TAT CGA GTC GAC AAA AAT CAG CAG AGA GCG CGA GGC AAC TAC
 161   L   R   Y   A   F   G   Y   R   V   D   K   N   Q   Q   R   A   R   G   N   Y

 541  GAG TTG CTG GGA CTG CTG ATC ATT TTT CAG ACA GTC TTC AAG AAC GTG GCA AAC TTG CGA
 181   E   L   L   G   L   L   I   I   F   Q   T   V   F   K   N   V   A   N   L   R

 601  AAA CTG TGG GGG GCC ACC AAG ACC GTG CAG GAC TCG GGA GAT TTA ATC TAC AGG TTC AGA
 201   K   L   W   G   A   T   K   T   V   Q   D   S   G   D   L   I   Y   R   F   R

 661  GAC CAG ACG AGC GAC GTT ATA GAC CTT GCA GAC CCA AAG GTG CTG CCG TAC CTG CCT GAG
 221   D   Q   T   S   D   V   I   D   L   A   D   P   K   V   L   P   Y   L   P   E

 721  GCT TCG CGC ACG TGC ATG CTG TGT CTG TCG CCG ATG AAA GAC CCA TCA TGC GGC GAG TGC
 241   A   S   R   T   C   M   L   C   L   S   P   M   K   D   P   S   C   G   E   C

 781  GGC CAC GTC TTC TGC TGG AAG TGC GTG CTG GAC TGG GTC AAG GAG CGC CAG GAG TGT CCG
 261   G   H   V   F   C   W   K   C   V   L   D   W   V   K   E   R   Q   E   C   P

 841  TTG TGC AGG GCC AAA ATG AGA GAG TCG CAG CTG TTG CCT CTA CGA TAA gtgtctatgaacaat
 281   L   C   R   A   K   M   R   E   S   Q   L   L   P   L   R  ***

 904  acataagggccacggatcagtttctattaacacccattcggaacagcacctgcttgcccttcttgccctttctgatctc
 983  gaagtcgggctcttcgcgtcgttgctgtaagccctgaccccattgtccgatcgaggtgttgaccgggttgacccttggt
1062  ggcttgggtttctgggcactgggcagtttcgggaacagctggtcgtccagagccggggcttgtggcttgggctgctaat
1141  tgttgaggtacgtcagccgcacgtttggcgaggtgcctctattgacgacaggcttcaacttcgggggcgcgctttgcgc
1220  agccccgttgtgggctggttctggccccacggtcccacgggccgcccgtggcgcagtgctgagcgcagggaactgctcg
1299  tccagcagcttttcg
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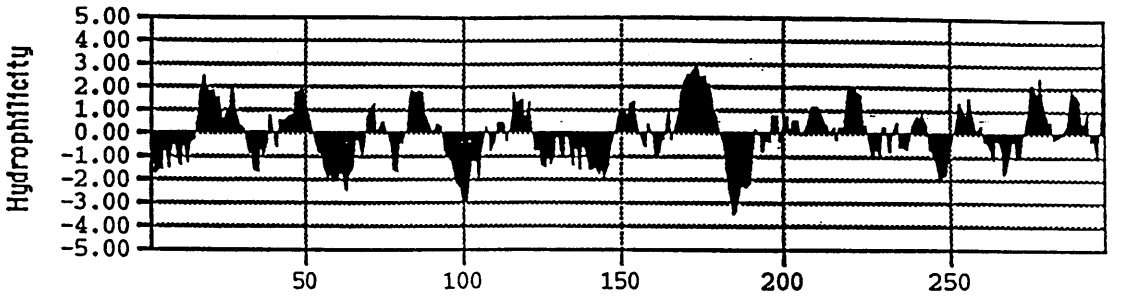
B



C

Per8p	SRTCMLC	LSPMKDPSCGE	CGHVFCWKCVLD	WVKERQ	ECPLCRAK
Pas4p	SRKCILC	LMNMSDPSCAP	CGHLFCWSCLMS	WCKERP	ECPLCRQH
PAF-1p	GKECALCGEWPTMPHTIG		CEHIFCYFCAKS	SFLFDVY	FTCPKCGTE
Pep5p	NQTCFMCRLTLDIPVVFFK		CGHIYHQHCLNEEEDTLESERKLFKCPKCLVD		
PS 43K	ELYCGLCGESIGERNSRLQALPCSHIFHLRCLQ			NNGTRS	CPNCRRS
consensus	CXIC	11-30X	CXHXFCXXCL	10-18X	CPXC
	V		YH I		
	L		L		

Figure 3.6. Analysis of *PER8* DNA and protein sequences. (*A*) Nucleotide and predicted amino acid sequences of *PER8*. The cysteine-rich motif is located from residue 245 through 282 in Per8p and is underlined. (*B*) Hydrophilicity plot of Per8p-predicted primary structure shows that the peptide is of average hydrophobicity and lacks potential a-helical membrane-spanning domains. (*C*) Alignment of C3HC4 motifs in selected proteins. Postsynaptic 43K protein, PS 43K.

fragment containing the *S. cerevisiae LEU2* gene. This plasmid was then digested with a restriction enzyme to release the *PER8*-deletion allele (*per8Δ*) on the linear DNA fragment shown in Figure 3.7A, and introduced into *H. polymorpha* strain A16 (*leu1*). Transformants in which the fragment had deleted the *PER8* locus were isolated by selecting for Leu^+ colonies and then screening for ones which were also Mut^-. Proper targeting of the fragment was confirmed by Southern blot analysis (Figure 3.7B). For example, P2, a labeled probe composed of sequences located entirely within the deleted region, hybridized to a 8.5-kb fragment from wild-type *H. polymorpha* but did not hybridize with DNAs from *per8Δ* strains (Figure 3.7B, P2 lanes 2 and 3). Furthermore, northern blots showed that methanol-induced cells of *per8Δ* strains no longer produced the 1.4-kb *PER8* message (Figure 3.5, lane 2). One *per8Δ*-derived strain, CT102 (*per8Δ::SLEU2 leu1 ade11*), was crossed with CT100 (*per8-1 leu1*) by selection for growth on minimal glucose plates. The resulting diploids were then tested for methanol growth and were Mut^-. In contrast, diploids from a control cross of CT100 and CT104 (*per1 leu1*) were Mut^+. The *per8-1/per8Δ* diploid strains were sporulated and several thousand of their progeny were grown on glucose medium and tested by the replica plate technique for methanol growth. All spore progeny were Mut^-, indicating that *per8-1* and *per8Δ* were tightly linked. The failure of *per8-1/per8Δ* diploids or their progeny to grow on methanol demonstrated that *per8-1* and *per8Δ* were likely to be mutant alleles of the same gene and therefore, that the cloned DNA fragment under investigation contained the same gene that was defective in *per8-1* and not a suppressor gene.

Phenotypically, the *per8Δ* strain was indistinguishable from *per8-1*. In addition to being Mut^-, EM examination of methanol-induced *per8Δ* cells showed that they were devoid of peroxisomes and contained the cytosolic AOX crystalloid that is typical of

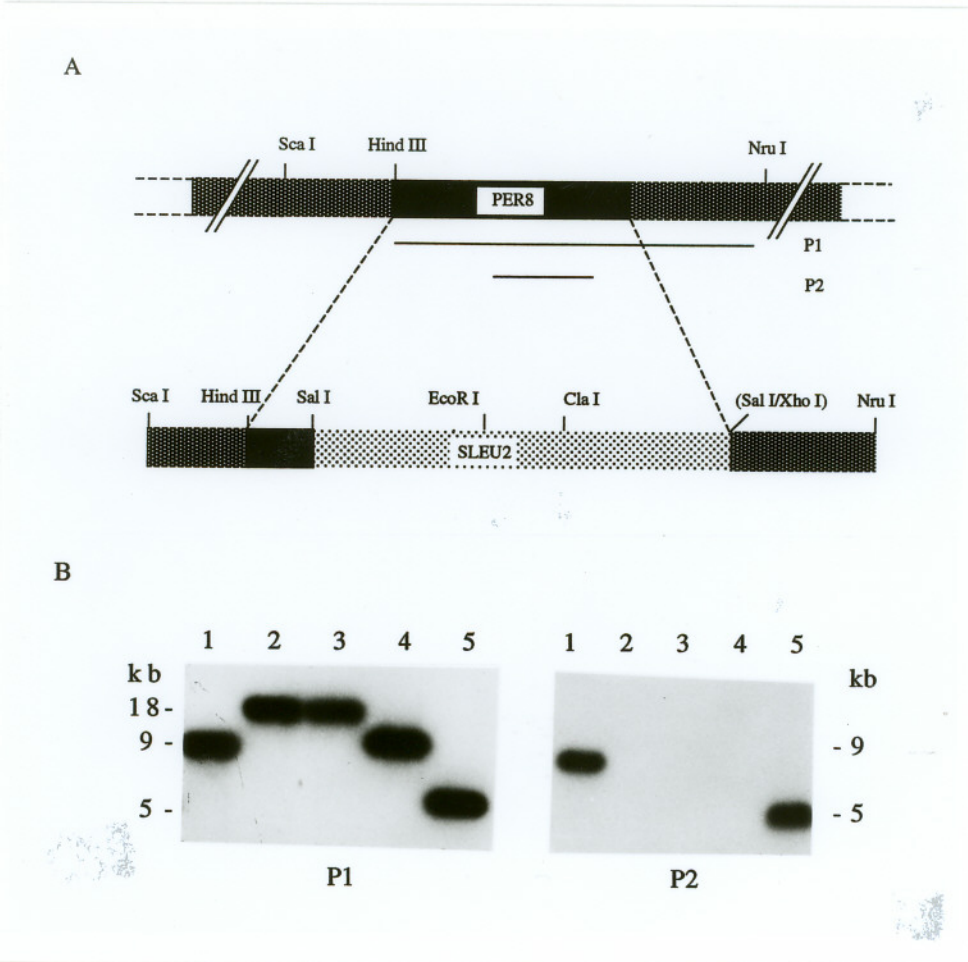


Figure 3.7. Deletion of the *PER8* gene. (*A*) *PER8* DNA sequences encoding Per8p amino acids 69 through 295 were replaced with a DNA fragment containing the *S. cerevisiae LEU2* gene and inserted into the *H. polymorpha* genome by homologous recombination. (*B*) Correct targeting of the *per8Δ* fragment was demonstrated by Southern blotting of genomic DNAs cut with NruI and hybridizing with labeled fragments P1 and P2 indicated in *A*. (lanes 1) 2 mg of wild-type genomic DNA; (lanes 2 and 3) 2 mg of DNA from each of two *per8Δ* strains; (lanes 4) 2 ng of linear DNA from *per8Δ* plasmid pDT6; (lanes 5) 2 ng of linear DNA from *PER8* plasmid pBS1.

H. polymorpha per mutants (not shown). In addition, peroxisomal enzymes in *per8Δ* cells were induced to normal levels but were mislocalized to the cytosol as judged by subcellular fractionation results (Figure 3.2).

3.4.4 Per8p overexpression leads to enhanced peroxisome proliferation

To gain insight into Per8p function, the effect of overexpression of *PER8* on the cell's morphology was examined. For these experiments, an *H. polymorpha* strain was constructed that expressed *PER8* under the transcriptional control of the methanol-regulated and highly efficient alcohol oxidase promoter (P_{MOX}). In methanol-grown cells of this strain (CT103; *leu1 per8-1* P_{MOX}*-PER8*), the level of *PER8* message was approximately 10 times higher than in wild-type cells (Figure 3.5, lanes 1 and 3). As described in the following section, immunoblots showed a similar increase in the level of Per8p as well. Expression from P_{MOX} is highly repressed by glucose; therefore, as expected, most glucose-grown CT103 cells contained only a single small peroxisome (Figures 3.8A and 3.9), similar to that in wild-type cells.

Detailed EM examination of P_{MOX}*-PER8* cells harvested at selected times after methanol induction revealed an interesting temporal series of events. Within the first few hours after shift, the single peroxisome enlarged several-fold (Figures 3.8B and 3.9), a phenomenon also observed in wild-type cells and due to the import of newly synthesized AOX, CAT and DHAS (Veenhuis and Harder, 1991). Between 4 to 6 hours post-shift, an enhanced rate of peroxisome proliferation was observed, resulting in the development of 5 to 10 new organelles (Figures 3.8C and 3.9), where, in wild-type cells, on average two new ones were formed. The newly formed peroxisomes consistently appeared at a single location on the parent organelle and often in close association with endoplasmic reticulum (Figure 3.10A).

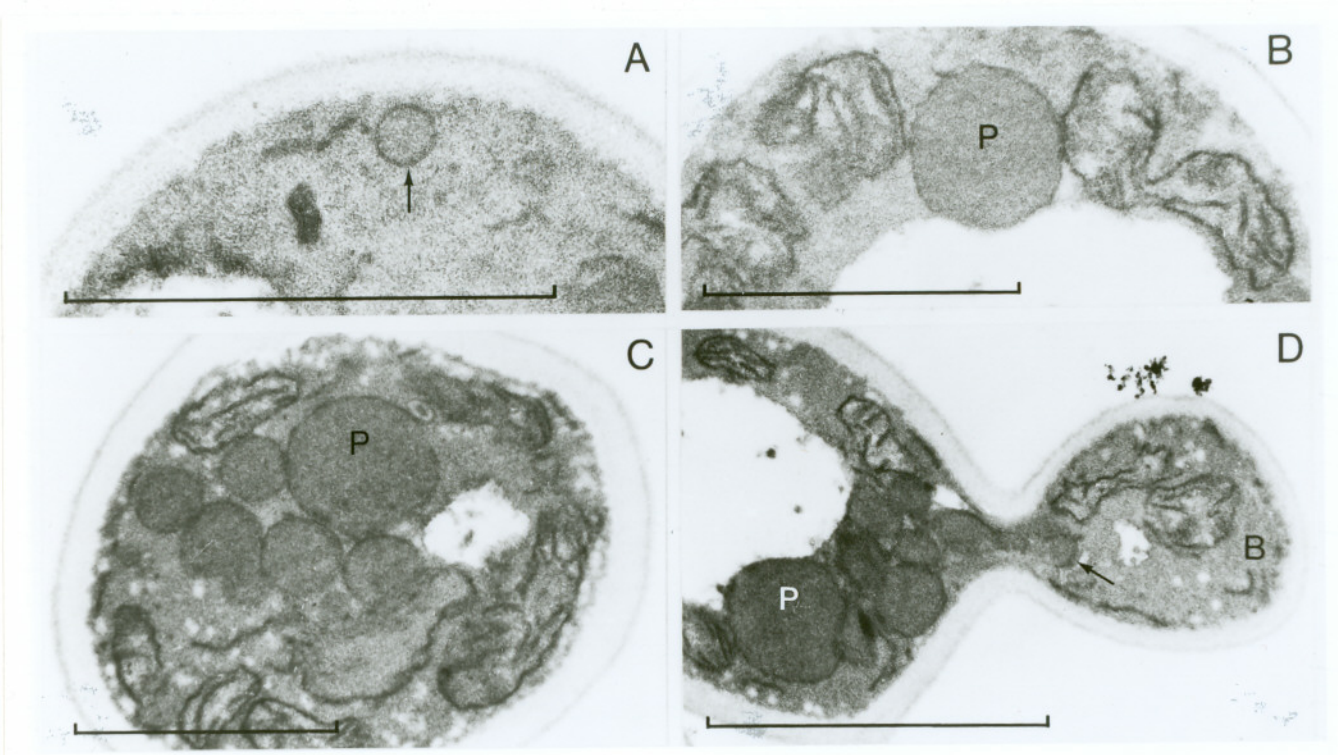


Figure 3.8. Electron micrographs showing enhanced peroxisome proliferation in P_{MOX}-*PER8* strain. (*A*) Single small peroxisome in glucose-grown P_{MOX}-*PER8* cell. (*B*) Three hours after shift to methanol medium peroxisome has grown but not proliferated. (*C*) Enhanced proliferation in cells after 6 hours in methanol. (*D*) Segregation of peroxisomes to bud at 6 hours.

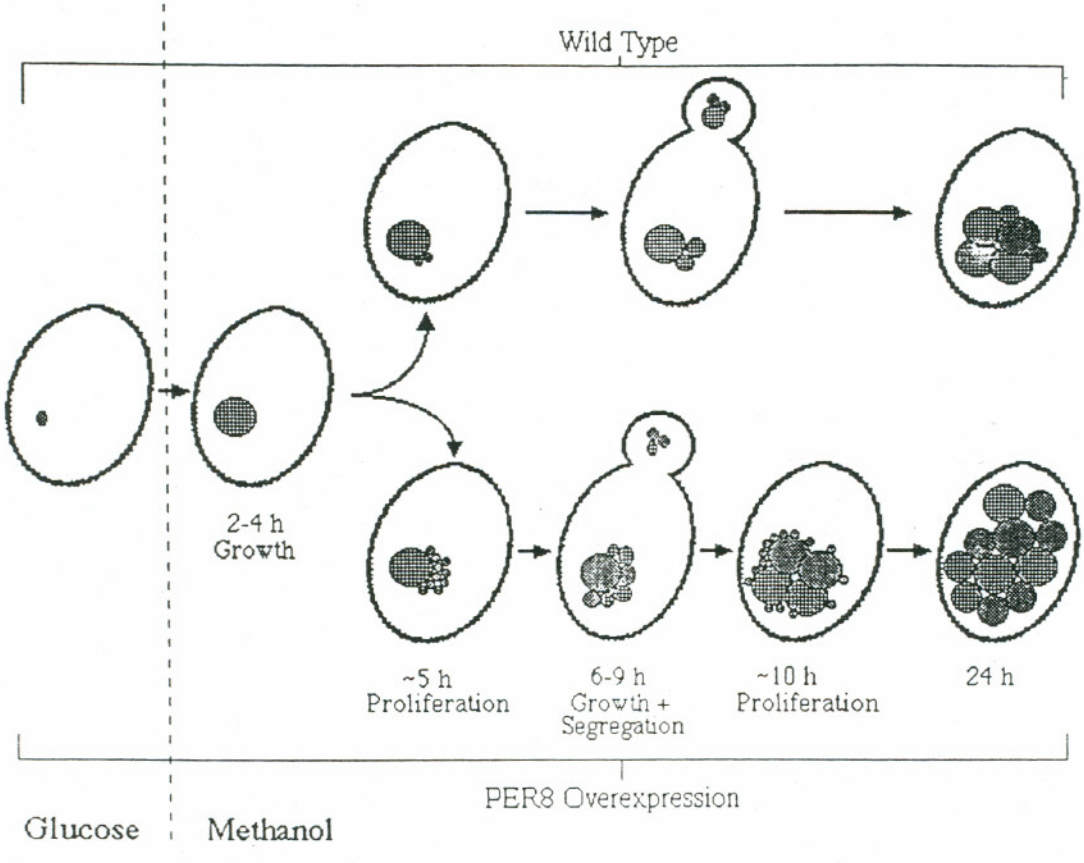


Figure 3.9. Diagram of peroxisome proliferation events in P_{MOX}-*PER8* overexpression strain and wild-type *H. polymorpha* after shift to methanol medium.

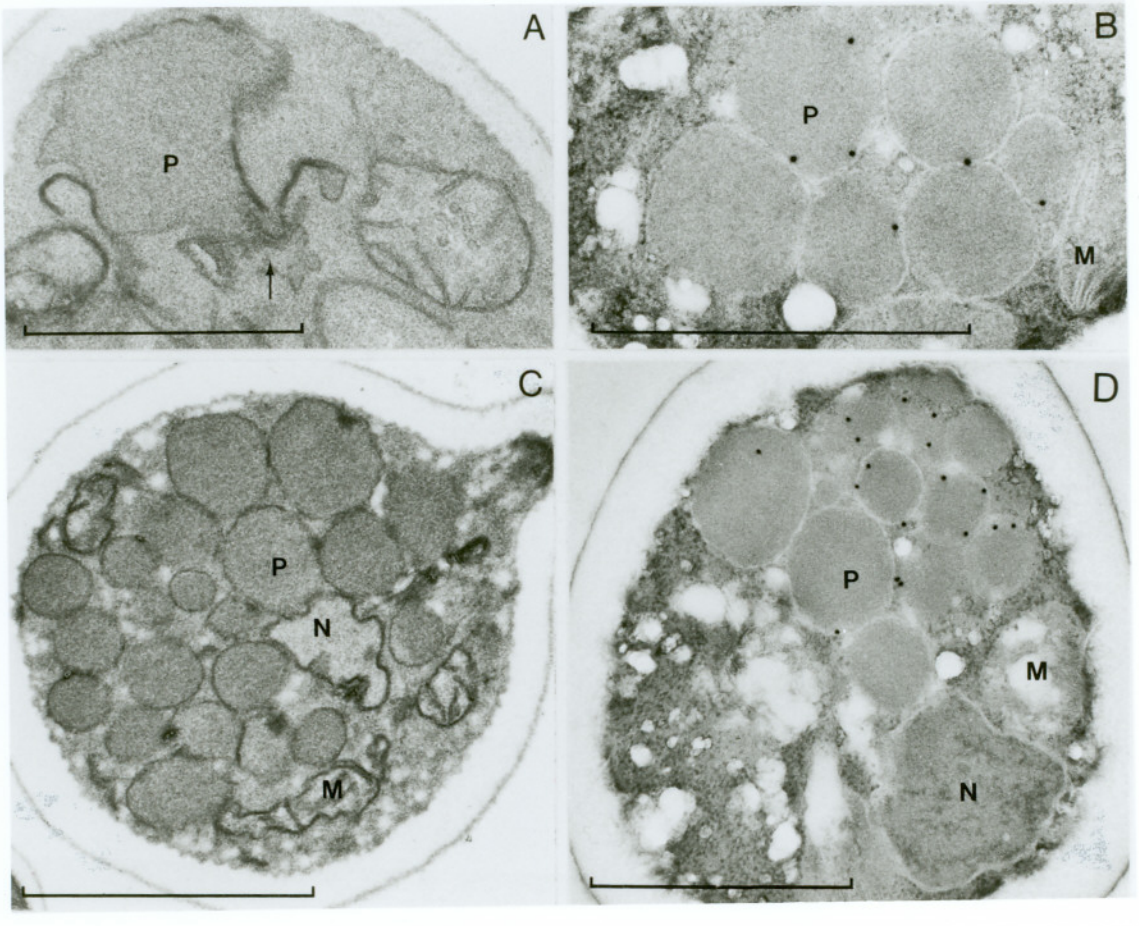


Figure 3.10. Per8p is involved in peroxisome proliferation. (*A*) Electron micrograph showing possible peroxisome fission event in the P_{MOX}-*PER8* strain 4 hours after shift to methanol medium. Subcellular localization of Per8p in methanol-grown wild-type (*B*) and P_{MOX}-*PER8* strain (*D*) after immuno-gold labeling using antibodies against Per8p. (*C*) P_{MOX}-*PER8* cell after 24 hours in methanol medium showing abnormally large number of peroxisomes.

Over the next several hours, the small newly formed organelles increased in size and some appeared to migrate into newly formed cell buds (Figures 3.8D and 3.9). However, the number of peroxisomes migrating into buds of the Per8p overexpression cells was not significantly greater than that observed in wild-type cell buds. Between 8 and 10 hours post-shift, a second proliferation round was observed that appeared to originate from one or two of the organelles that had been generated during the first round. This led to a further increase in peroxisome numbers (Figures 3.9 and 3.10C). Thus, the increased proliferation of peroxisomes in response to Per8p overexpression indicated that Per8p plays an important role in regulating the number of peroxisomes generated during proliferation. That proliferation did not occur continuously suggested that other factors determine the timing of these events.

3.4.5 Per8p is a peroxisomal integral membrane protein

Per8p was characterized through rabbit polyclonal antibodies raised against Per8p expressed in *E. coli* as a fusion with maltose binding protein. The crude anti-Per8p antibody serum recognized a 31-kD polypeptide in immunoblots prepared from methanol-grown wild-type and *per8-1* extracts that was not present in extracts from methanol-induced *per8Δ* cells (Figure 3.11A, lanes 1, 2 and 4). The apparent molecular weight of Per8p was in reasonable agreement with the 34-kD weight calculated from the predicted primary sequence. The putative Per8p band was difficult to detect, suggesting that Per8p is a low abundance protein even in methanol-grown *H. polymorpha* where peroxisomes are prominent (Figure 3.1A). To confirm the identity of the 31-kD species as Per8p, extracts prepared from methanol-grown cells of the P_{MOX}-*PER8* strain were examined and showed a very prominent band at 31 kD (Figure 3.11A, lane 3). Subsequent affinity purification of the serum resulted in a preparation that reacted specifically with

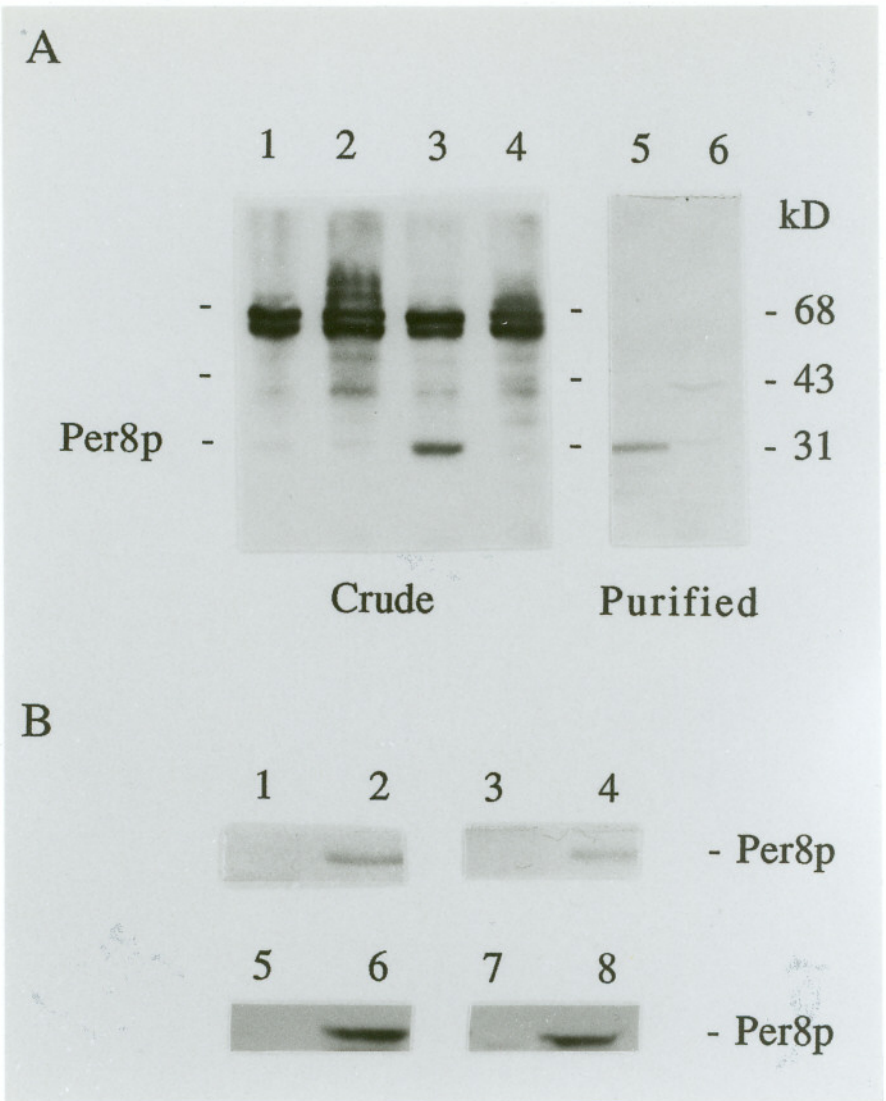


Figure 3.11. Per8p is a peroxisomal integral membrane protein. (*A*) Immunoblots showing that both crude and affinity-purified anti-Per8p antisera react with a 31-kD protein species in extracts prepared from methanol-induced cells. Lanes 1 through 4 each contain 10 mg of protein extract, and lanes 5 and 6 each contain 30 mg of protein extract from the following *H. polymorpha* strains: (lane 1) wild type; (lane 2) *per8-1* strain; (lanes 3 and 5) P_{MOX}-*PER8* strain; (lanes 4 and 6) *per8Δ* strain. (*B*) Distribution of Per8p after extraction of peroxisomes. In lanes 1 through 4, 30 mg of purified peroxisomes isolated from methanol-grown wild-type cells were extracted with 20 mM triethanolamine, pH 7.8, (lanes 1,2) or 0.1 M sodium carbonate, pH 11, (lanes 3,4). In lanes 5 through 8, 28 mg of post-differential centrifugation pellet fraction from methanol-grown P_{MOX}-*PER8* cells were extracted with 0.5% Triton X-114 (lanes 5 and 6) or 6 M urea (lanes 7 and 8). After extraction, samples were centrifuged, and supernatant (odd numbered lanes) and pellet (even numbered lanes) fractions (for Triton extractions, aqueous and detergent phase fractions) were immunobloted with anti-Per8p antibodies.

Per8p (Figure 3.11A, lanes 5 and 6).

To determine the subcellular location of Per8p, immunocytochemical experiments were performed on sections from methanol-grown cells of wild-type and *P_{MOX}-PER8* strains. In both, specific labeling was found almost exclusively on or near the peroxisomal membranes (Figure 3.10B and D). Moreover, the gold particles appeared to be concentrated on the membranes of small (presumably newly formed) organelles, a feature that was particularly evident in *P_{MOX}-PER8* cells (Figure 3.10D).

The location of Per8p on or near peroxisomal membranes was characterized biochemically. Peroxisomes from methanol-grown wild-type cells that had been purified by sucrose density gradient centrifugation were examined by immunoblotting and were found to contain Per8p. Samples of these purified peroxisomes were extracted with either triethanolamine or sodium carbonate. Following triethanolamine treatment and centrifugation at 30,000 x g, AOX and CAT were located mostly in the supernatant, whereas Per8p was found in the membrane pellet (Figure 3.11B, lanes 1 and 2), suggesting that Per8p was associated with the peroxisomal membrane. After carbonate extraction and centrifugation at 100,000 x g, Per8p remained in the membrane pellet (Figure 3.11B, lanes 3 and 4), suggesting that Per8p was an integral peroxisomal membrane protein. To further support this conclusion, samples of pellet fractions resulting from differential centrifugation of methanol-grown *P_{MOX}-PER8* cells were subjected to extraction with urea or Triton X-114. After Triton X-114 extraction and centrifugation at 300 x g, Per8p partitioned from the aqueous phase to the detergent phase as expected for a membrane protein (Figure 3.11B, lanes 7 and 8). After urea extraction and centrifugation at 250,000 x g, Per8p was mostly in the membrane pellet (Figure 3.11B, lanes 5 and 6).

3.5 Discussion

Previously, we described the isolation and preliminary characterization of the chemically induced *per8-1* strain as one of a large number of *H. polymorpha* mutants affected in peroxisome biogenesis (Cregg et al., 1990; Waterham et al., 1992; Titorenko et al., 1993). In this report, we describe the further characterization of the *per8-1* strain and its comparison to an *in vitro*-constructed *per8Δ* null mutant. Both *per8-1* and *per8Δ* alleles result in *H. polymorpha* strains without any peroxisome-like structures, a phenotype we have named Per$^-$. Their apparent total absence contrasts with some mammalian peroxisome-deficient mutants where small abnormal vesicular structures, believed to be peroxisomal remnants, are observed (Santos et al., 1988). Consistent with the lack of peroxisomes in *per8* mutants, all peroxisomal matrix enzymes that we examined (AOX, CAT, DHAS and AMO) are located in the cytosol. To date, two peroxisomal targeting signals, PTS1 and PTS2, have been defined (de Hoop and AB, 1992; Subramani, 1993). The three methanol-pathway enzymes, AOX, CAT and DHAS, are believed to be imported via a PTS1-type system since each ends in a tripeptide with similarity to the prototypical PTS1, SKL (Didion and Roggenkamp, 1992; Hansen et al., 1992). Specifically, the carboxy-terminal amino acids are ARF for AOX, SKI for CAT, and NKL for DHAS. For each of these enzymes, it has been shown that deletion of the three terminal residues results in a mutant enzyme that is cytosolic. In addition, the carboxy-termini of AOX and DHAS were demonstrated to be sufficient for peroxisomal targeting when appended to a non-peroxisomal protein (Hansen et al., 1992). Thus, it appears that *per8* mutants cannot import PTS1-type enzymes. AMO is required for utilization of methylamine as a nitrogen source and has recently been shown to be imported via

an amino terminal PTS2 sequence (Faber and Veenhuis, unpublished results). Thus, the failure of *per8* mutants to import AMO suggests that PTS2-containing proteins are also not imported. Furthermore, the fact that methylamine-grown *per8* cells are also peroxisome-deficient indicates that this defect is not limited to the organelles of cells in methanol, but is a general one affecting peroxisome biogenesis under all conditions. Overall, our studies of *per8* mutants demonstrate that they are profoundly and specifically affected in peroxisome biogenesis and therefore, that the *PER8* gene is essential for this process.

To gain insight into the structure of the *PER8* gene product, the *PER8* locus was cloned. The sequence of Per8p predicts a novel polypeptide of 295 amino acids (~34 kD) with no overall sequence similarity to any other protein in the data bases. [However, the *S. cerevisiae* peroxisome biogenesis protein Pas4p shows strong similarity to Per8p over its entire length (38% identity and 50% similarity) suggesting it may be the functional homologue of Per8p (W.-H. Kunau, personal communication).] The carboxy-terminal region of Per8p contains a segment of 39 residues that is a perfect match to the C3HC4 motif, a subgroup within the cysteine-rich zinc-finger domain family (Freemont et al., 1991; Coleman, 1992). For most of the approximately 30 proteins in this group, the C3HC4 region is thought to be involved in nucleic acid binding. However, in no case has the binding of zinc by a C3HC4 motif actually been demonstrated, and DNA binding by the motif is only surmised from the protein's function, such as DNA repair (Rad18p; Jones et al., 1988), transcriptional regulation (PML; Kakizuka et al., 1991), or DNA binding (MEL-18; Tagawa et al., 1990). Per8p belongs to a subset of proteins within the C3HC4 subgroup whose members are not located in the nucleus and therefore, probably do not interact with DNA. These proteins include: the *S. cerevisiae* vacuolar membrane-associated proteins Vps11p/Pep5p/End1p (Dulic and Riezman,

1989) and Vps18p/Pep3p (Robinson et al., 1991); the mammalian peroxisomal integral membrane protein PAF1p (Shimozawa et al., 1992); and the mouse peripheral membrane postsynaptic 43-kD protein (Froehner, 1989). These proteins are further distinguished from other C3HC4 subgroup members by the carboxy-terminal location of the motif. It has been suggested that the C3HC4 motif in these non-nuclear proteins is involved in protein-protein interactions (Freemont et al., 1991; Kunau et al., 1993). With regard to Per8p, this hypothesis is consistent with recent genetic evidence that the *PER8* gene product interacts with at least three other *PER* products (Titorenko et al., 1993).

Per8p behaves in biochemical experiments as a peroxisomal integral membrane protein. After discontinuous sucrose centrifugation of crude organelle preparations, Per8p is present in peroxisomal fractions. Furthermore, after extraction with sodium carbonate, urea or Triton X-114, Per8p remained associated with the membrane fraction, indicating that Per8p is an integral membrane protein. These results were further supported by immunocytochemical experiments which showed that Per8p is exclusively located on the peroxisome membrane.

The failure of *per8* mutants to import peroxisomal enzymes might be taken as evidence that Per8p is a component of the peroxisome protein import machinery. However, defects in other peroxisome functions such as the duplication, proliferation or segregation should also result in the absence of peroxisomes and, as a consequence, a cytosolic location for peroxisomal enzymes. An important clue to the possible function of Per8p comes from ultrastructural studies of an *H. polymorpha* strain that overexpresses *PER8* under control of the alcohol oxidase promoter P_{MOX}. Upon shift of this strain from glucose to methanol, a greatly enhanced proliferation of peroxisomes occurs. The extent of this proliferation is not seen in wild-type cells and thus, appears to be a response to the increased

amount of Per8p. The proliferation events in the overexpression strain take place in bursts at approximately 5 and 10 hours after shift. Such synchronous bursts are not observed in wild-type cells and are not readily explained. Perhaps in wild-type cells, the concentration of Per8p is normally the limiting factor controlling the initiation of proliferation. When Per8p is overexpressed, another factor becomes limiting and thereby, determines the timing of proliferation events. One possibility for a factor that might synchronize proliferation is suggested by the observation that peroxisomes appear to need to reach a critical volume or mature size before budding begins (Veenhuis and Harder, 1991). Thus, a factor that senses peroxisome volume may be limiting. A second possibility is suggested by the time between proliferation cycles which is approximately the same as the generation rate of the strain in methanol medium. Thus, a factor that links proliferation to the cell cycle may be limiting initiation events in the Per8p overexpression strain. Most importantly, the hyperproliferative response of peroxisomes to high levels of Per8p indicates that the protein is a component of the machinery that controls peroxisome proliferation in this yeast.

A role for Per8p in proliferation is also supported by our observation that the protein is concentrated in the membranes of small and presumably newly formed peroxisomes. Peroxisome budding is similar in overall appearance to the formation of vesicles during endocytosis and secretion. During the latter vesicle formation events, components of the fission machinery are known to concentrate in or around the membranes of emerging vesicles (Pryer et al., 1992). Although the mechanisms driving peroxisome and secretory-vesicle budding may be very different at the molecular level, it is likely that both involve the presence of specific machinery components at the site of the emerging vesicle. Therefore, the presence of Per8p predominantly in newly formed peroxisomes relative to larger older

organelles is consistent with the involvement of the protein in peroxisome proliferation.

Several considerations lead us to suspect that Per8p may be involved in at least one other aspect of peroxisome biogenesis. If Per8p was solely involved in the proliferative response to environmental changes but not in the peroxisome duplication or segregation processes responsible for maintaining the organelle through cell division cycles, *per8* cells would be predicted to continue to contain at least one small peroxisome. Upon methanol induction, one would expect the small organelle to increase in size through import of matrix proteins. This expectation comes from studies on the effect of expressing AOX and DHAS in glucose-grown *H. polymorpha*. When either of these enzymes are expressed at a high level in glucose medium, the one or two small peroxisomes that normally exist in these cells are observed to increase in volume but not number (Distel et al., 1988; Godecke et al., 1989; Roggenkamp et al., 1989). The same effect, peroxisome growth without proliferation, would also be expected of a mutant that is specifically defective in proliferation. In contrast, *per8* cells appear to have no peroxisomes and, upon methanol induction, peroxisomal enzymes are located in the cytosol. This suggests that, in addition to environmentally induced proliferation, Per8p is also required for some other aspect of peroxisome biogenesis.

In conclusion, the hyperproliferative response of peroxisomes to *PER8* overexpression and the concentrated presence of Per8p in the membrane of newly formed organelles indicate that Per8p is part of the machinery controlling peroxisome proliferation in *H. polymorpha*. The similarity of Per8p to Pas4p, a protein required for peroxisome biogenesis and oleate growth in *S. cerevisiae* (W.-H. Kunau, personal communication), strongly suggests that these proteins are not pathway- or organism-specific products, but are a universal component of the organelle's proliferative machinery.

CHAPTER 4
CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Summary of Research

The long-term goal of this research is to understand, at molecular level, the mechanisms by which cells assemble and maintain peroxisomes, and direct specific sets of proteins to these organelles in *H. polymorpha*. As a first step, we identified a large collection of peroxisome-deficient (*per*) mutants from a collection of Mut$^-$ strains by direct electron microscopic examination. The *per* mutants represent mutations in 14 different *PER* genes. In methanol-induced cells of each mutant, peroxisomes appear to be absent and peroxisomal enzymes are mislocated in the cytosol. At present, the reason that peroxisomes are absent in *per* mutants is unknown. They could be defective in peroxisome fission, the segregation of the organelles to daughter cells, a protein recognition/import system or regulatory factors involved in expression of other products required for peroxisome biogenesis. Based on the results of this research and that of others (van der Klei et al., 1991), a more rapid and efficient screen for identifying *per* mutants has been identified. In the future, Mut$^-$ strains can be examined first by light microscopy for the presence of a large AOX crystalloid structure in each cell. This structure is easily observed and exists only in *per* mutants. Electron microscopy need only be performed to confirm a peroxisome-deficient diagnosis. We have also cloned and characterized *PER8*, a gene required for peroxisome biogenesis in *H. polymorpha*. We demonstrate that its gene product is a peroxisomal integral membrane protein and provide

evidence that it is involved in peroxisome proliferation. To our knowledge, this is the first description of a component of peroxisome's proliferation machinery.

4.2 Future Directions

Although we have shown that Per8p is a peroxisomal integral membrane protein and appears to play a role in peroxisome proliferation, there are still many unanswered questions about the role of this protein in peroxisome biogenesis. Additional experiments should be performed to confirm that Per8p is involved in proliferation and to characterize the mechanism(s) of Per8p action.

4.2.1 Per8p function

Additional support for the role of Per8p in peroxisome proliferation and possibly other peroxisomal functions such as import may be obtained through the examination of a temperature sensitive (ts) mutant in *PER8*. Such a strain could be generated by mutagenizing an *H. polymorpha* vector that contains the wild-type *PER8* gene, and screening *per8* cells transformed with the mutagenized vector population for ones which are ts for growth on methanol. Methanol-induced cells of candidate *per8*ts strains would then be analyzed biochemically and cytologically for one that contains normal peroxisomes at permissive temperature, but no peroxisomes at nonpermissive temperature.

To gain insight into the role of Per8p, the *per8*ts strain would be grown at permissive temperature on glucose and shifted to methanol medium. After approximately 3 hours on methanol, the culture could be further shifted to nonpermissive temperature. At this point, peroxisome proliferation will have just begun in these cells but little protein will have been imported. Therefore, these cells should appear to contain several small peroxisomes. After shift to nonpermissive temperature, samples would be removed at

selected times and examined for peroxisome morphology. If Per8p is involved in proliferation only, the few small peroxisomes should increase in size but not number. Conversely, if Per8p functions only in protein import, the organelles should appear to increase in number but not size. Finally, if Per8p is involved in both processes, both the size and number of peroxisomes should remain unchanged after shift to nonpermissive temperature.

The hyper-proliferation of peroxisomes in response to the over-expression of *PER8* in methanol suggests that Per8p may be a limiting factor in proliferation of the organelle. We would like to determine whether the concentration of Per8p controls peroxisome number under other growth conditions as well. To address this question, a strain in which an extra copy of *PER8* under the control of a promoter which is highly expressed in glucose grown cells could be constructed. When this strain is grown in glucose medium, Per8p but not other peroxisomal proteins would be synthesized in large amounts. If more than the one or two peroxisomes that are normally observed in wild-type glucose-grown cells are observed, it would suggest that the concentration of Per8p alone can control peroxisome number. On the other hand, it is possible that other factors, in addition to Per8p are needed for proliferation and that over-expression of *PER8* in glucose will not lead to increased numbers of organelles.

4.2.2 Per8p topology

We have demonstrated that, despite a lack of an apparent membrane spanning domain in its sequence, Per8p behaves as an integral membrane protein. We would like to determine how Per8p is oriented in the membrane. Initial information on this question can be obtained from experiments in which intact peroxisomes are incubated with proteinase K and the effect on Per8p will be observed by immunoblotting. If most of Per8p faces the cytosol, addition of proteinase K will result in degradation (or at least partial degradation)

of Per8p. If most of Per8p faces the peroxisomal matrix or is embedded in the membrane, Per8p will be not be significantly degraded since the proteinase will not penetrate the peroxisomal membrane. As a control, samples treated with Triton X-100 to lyse peroxisomes and thereby, expose inner membrane proteins to proteinase K will be examined as well.

Additional insight into Per8p orientation can be gained by examining peroxisomes from cells expressing epitope-tagged derivatives of Per8p. Sequences encoding *myc* or other tags will be inserted into *PER8* the extreme 3' and/or 5' end of Per8p encoding sequences and the resulting tagged derivatives of *PER8* transformed into a *per8* mutant strain. If either or both tagged *PER8* derivatives complement the mutant for growth on methanol and for the restoration of normal peroxisomes, the resulting strains will be used as a source of peroxisomes for additional protease digestion studies. By determining the size of Per8p-derived peptide fragments generated upon incubation of peroxisomes with proteinase, we should be able to estimate the location of cleavage sites within Per8p and therefore, the location of regions of Per8p facing the cytosol. In addition, it is expected that the monoclonal antibodies against the tag sequence will be more specific and sensitive than our anti-Per8p antibodies and therefore, allow us to more easily observe Per8p in organelle preparations.

We would also like to determine which sequences target Per8p to the peroxisomal membrane. Although two types of targeting signals have been described in detail for peroxisomal matrix proteins, a targeting signal for a peroxisomal membrane protein has yet to be defined. To locate the membrane targeting sequence, a series of deletions in epitope-tagged *PER8* would be generated and inserted into wild-type *H. polymorpha*. The fate of these truncated Per8p products would then be examined by differential centrifugation or by indirect immunofluorescence microscopy. Since peroxisomal targeting of Per8p is likely to require both a targeting signal and at least one membrane

spanning domain, these experiments may result in the identification of a targeting signal that is either within a membrane spanning domain or is soluble but requires a membrane domain for the protein to insert.

As described in Chapter 3, the C-terminal region of Per8p has strong similarity to the C3HC4 motif, a subgroup within the cysteine-rich zinc-finger domain family. However, binding of zinc by a C3HC4 motif has never been directly demonstrated. To address this question, Per8p could be translated *in vitro* and its ability to bind zinc assayed. If Per8p appears to bind zinc, the importance of the C3HC4 domain in zinc binding could be determined by *in vitro* translating a C3HC4 peptide and ascertaining whether the peptide also binds zinc. The role of specific cysteines in zinc-binding could be determined by altering the sequence encoding each cysteine by site-directed mutagenesis and determining the effect of each change on zinc binding. Finally, those cysteine positions that appear to be important for zinc binding *in vitro* can be examined for their importance to peroxisome biogenesis *in vivo* by expressing appropriate mutant *PER8* genes in a *per8Δ* strain and examining the resulting strains, for ability to complement *per8Δ* for methanol-growth phenotype and restoration of functional peroxisomes.

4.2.3 Interaction of Per8p with other proteins

Many proteins essential for peroxisome biogenesis are likely to operate as components in multi-subunit protein complexes. In *H. polymorpha*, genetic studies have indicated that Per8p interacts with protein products of *PER1, PER2* and *PER5* (Titorenko et al., 1993). Per1p has been shown to be a peroxisomal matrix protein that appears to be involved in protein import into peroxisomes (Waterham et al., 1994). Since antibodies against both Per1p and Per8p are available, it should possible to detect their physical interaction in immunoprecipitation studies. Antibodies against Per8p could be utilized to

precipitate complexes that contain Per8p and the precipitated protein complexes examined for Per1p by immunoblotting with antibodies against Per1p. As antibodies against additional PER products become available, Per8p-containing complexes can by examined for these products as well.

A more general approach toward identifying proteins that interact with Per8p is possible utilizing the two hybrid protein approach. A DNA segment encoding a soluble portion of Per8p could be inserted into a vector that synthesizes Per8p peptide as a fusion with the DNA-binding region of the *S. cerevisiae* galactose-utilization regulatory protein ($Gal4p_{DB}$). A library of random genomic *H. polymorpha* DNA fragments would then be created in a *S. cerevisiae* vector that expressed these fragments as a fusion with the transcriptional activator region of Gal4p ($Gal4p_{TA}$). The library would be transformed into an *S. cerevisiae* strain that expressed the $Gal4p_{DB}$-Per8p fusion and also contained a *HIS3* gene under control of cis-acting *GAL4* regulatory sequences. Interaction of $Gal4p_{DB}$-Per8p with a protein fused to $Gal4p_{TA}$ would result in recruitment of the *GAL4* regulatory sequences, which would activate transcription of *HIS3* and result in transformants which are His^+. The His^+ transformants could be easily selected on a medium without histidine. DNA fragments whose products are candidates for interaction with Per8p would be recovered from *S. cerevisiae* cells and their DNA sequences as well as that of their products analyzed. Antibodies against potential Per8p-interacting proteins would be generated and used in immunoprecipitation experiments to confirm that genes isolated by the two hybrid technique in fact encode products that interact with Per8p. Finally, DNA fragments which encode Per8p-interacting proteins would be utilized to construct strains in which these genes are deleted and the resulting deletion strains would be examined to determine whether they are *per* mutants, indicating that the newly isolated genes, like *PER8*, are essential for peroxisome biogenesis.

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