ISOLATION AND CHARACTERIZATION OF PEROXISOME-DEFICIENT MUTANTS AND A PEROXISOME BIOGENESIS GENE FROM THE METHYLOTROPHIC YEAST *PICHIA PASTORIS*

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

ISOLATION AND CHARACTERIZATION OF PEROXISOME-DEFICIENT MUTANTS AND A PEROXISOME BIOGENESIS GENE FROM THE METHYLOTROPHIC YEAST *PICHIA PASTORIS*

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This thesis describes the isolation and characterization of peroxisome-biogenesis-defective (*per*) mutants of *Pichia pastoris* as well as the cloning and analysis of one of the affected genes called *PER3*. To isolate *per* mutants, a collection of 280 methanol-utilization defective (Mut^-) mutants was organized into 46 complementation groups. Ten of these groups were also unable to utilize oleate (Out^-) but still able to grow on glucose, ethanol and glycerol, the phenotype expected of *per* mutants. Electron microscopic examination indicated that mutants in 8 of these groups lacked normal-appearing peroxisomes. Consistent with the absence of peroxisomes, differential centrifugation results indicated that the peroxisomal marker enzyme catalase was mislocalized to the cytosol in each *per* mutant.

One mutant, *per3-1*, was used to clone *PER3* by functional complementation. *PER3* transcribed a message of 2.1 kb that is constitutively expressed but at a higher level in methanol-grown cells. The DNA sequence of *PER3* revealed an open reading frame of 2139 bp with the potential to encode a protein of 713 amino acids (81 kD). The putative *PER3* product (Per3p) contains a variant of the peroxisomal targeting signal PTS1 (AKL)

at its carboxy terminus. Biochemical studies indicated that Per3p is a peroxisomal membrane protein. Studies of *per3-1* cells showed that the bulk of PTS1-containing proteins are present in the cytosol while the PTS2-containing enzyme thiolase is imported normally into small peroxisomal remnants. However, a mutant strain in which most of the *PER3* had been deleted failed to import either PTS1- or PTS2-containing proteins. We hypothesize that Per3p is a component of the *P. pastoris* peroxisomal protein import machinery and that it may be composed of multiple domains involved in the import of specific groups of peroxisomal matrix proteins.

CHAPTER 1

INTRODUCTION

1.1 Discovery of Microbodies

Compartmentalization of metabolic pathways is a distinctive feature of eukaryotic cells. The microbody is one class of compartments that was first reported in 1954 by Rhodin in mouse kidney cells as a single membrane-bound vesicle with a diameter of approximately 0.5 μm (Rhodin, 1954). In 1960, de Duve and colleagues confirmed the existence of microbodies in rat liver and identified catalase, uricase (urate oxidase) and D-amino acid oxidase as enzymes located in this organelle (de Duve et al., 1960). Since these oxidases produce hydrogen peroxide, they gave microbodies the functional name peroxisomes (de Duve and Baudhuin, 1966). Later, Breidenbach and Beevers showed that in germinating seeds, two key enzymes of the glyoxylate pathway, malate synthase and isocitrate lyase, were present in a single membrane-bound organelle that they termed the glyoxysome (Breidenbach and Beevers, 1967). Enzymes for the ß-oxidation pathway are also present in this organelle, enabling germinating seeds to convert lipids to carbohydrates (Cooper and Beevers, 1969). Another type of microbody was discovered in the African trypanosome *Trypanosoma brucei*. This parasitic protozoan belongs to the order Kinetoplastida and is responsible for sleeping sickness in humans and a similar disease called Nagana in cattle (Opperdoes, 1988). First described by Opperdoes and Borst, the organelle contains nine enzymes for glycolysis, and therefore is called a glycosome (Opperdoes and Borst, 1977). Recently, it has become apparent that all these organelles are members of the microbody/peroxisome family (Keller et al., 1991).

1.2 Characteristics of Microbodies

Virtually all eukaryotic cells contain microbodies (Subramani, 1993). Morphologically, in addition to being enclosed within a single membrane, they have a granular matrix, sometimes with a crystalline core. Enzymatically, microbodies typically contain at least one H_2O_2-generating oxidase, along with catalase to decompose H_2O_2 to water and oxygen (de Duve and Baudhuin, 1966). Microbodies have no DNA (Kamiryo et al., 1982); therefore it is likely that all peroxisomal proteins (both matrix and membrane) are encoded by nuclear genes (Lazarow and Fujiki, 1985). Proteins destined for peroxisomes are synthesized on cytosolic polysomes and post-translationally imported into the organelle (Lazarow and Fujiki, 1985). It is thought that peroxisomes are derived from pre-existing peroxisomes (Lazarow and Fujiki, 1985), although recent evidence indicates that under certain circumstances they may arise *de novo* (Waterham et al., 1993). An unusual feature of peroxisomes is that the specific metabolic pathways present in the organelles vary depending upon the organism, cell type, and metabolic needs of the organism (van den Bosch et al., 1992). In addition to differences in enzymatic content, both the size and number of peroxisomes can change dramatically in response to environmental stimuli (Veenhuis and Harder, 1987; Veenhuis et al., 1983; Zwart et al., 1983b).

1.3 Microbody Metabolic Pathways

1.3.1 ß-oxidation of fatty acids

A peroxisomal ß-oxidation system in rat liver was first described by Lazarow and de Duve (1976). Further work demonstrated that this pathway exists in the peroxisomes of

almost all cells including those of mammals, plants, yeasts and trypanosomes (Opperdoes, 1987; Tolbert, 1981; Kunau et al., 1987; van den Bosch et al., 1992). In fact, fatty acid ß-oxidation is exclusively a peroxisomal event in most organisms except mammals which degrade fatty acids in their mitochondria. Mammals use two ß-oxidation systems: a mitochondrial pathway responsible for degrading fatty acids up to a chain length of C_{22}, and a peroxisomal pathway that degrades very-long-chain-fatty acids (C_{24} and greater) (Lazarow, 1988).

Peroxisomal ß-oxidation pathway enzymes include: an acyl-CoA synthase located on the peroxisomal membrane that forms acyl-CoAs; an H_2O_2-generating acyl-CoA oxidase that oxidizes acyl-CoAs to enoyl-CoAs; a bifunctional enzyme with enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase activities that convert enoyl-CoAs to 3-hydroxyacyl-CoAs and 3-ketoacyl-CoAs, respectively; and 3-ketoacyl-CoA thiolase that catalyzes the thiolytic cleavage of 3-ketoacyl-CoAs to form acyl-CoAs (shortened by two carbon units) and acetyl-CoA. The ß-oxidation cycle is then repeated to further shorten acyl-CoAs (Kunau et al., 1988; Kunau et al., 1987; Lazarow, 1988).

Interesting variations of the ß-oxidation pathway are known. In *Saccharomyces cerevisiae*, the pathway follows a recently discovered course involving a D-3-hydroxyacyl-CoA intermediate instead of L-3-hydroxyacyl-CoA (Hiltunen et al., 1992). In the glycosomes of *Trypanosoma.brucei*, catalase is absent and an acyl-CoA dehydrogenase rather than acyl-CoA oxidase catalyzes the formation of enoyl-CoA (Borst, 1989).

Acetyl-CoA produced from ß-oxidation can either remain in the microbody, where it enters the glyoxylate cycle in a process that generates carbon skeletons for cell growth, or move to the mitochondrion where it enters the tricarboxylic acid cycle to generate energy (Veenhuis and Harder, 1987).

1.3.2 Glyoxylate cycle

During the germination of fatty seeds, a portion of the acetyl-CoA generated by ß-oxidation is converted to succinate in microbodies via enzymes of the glyoxylate pathway (Tolbert, 1981). The succinate is then used for the biosynthesis of all other cellular components, especially carbohydrates. Because key enzymes of the cycle, isocitrate lyase and malate synthase, are present in the microbodies, the organelles in fatty seeds are called glyoxysomes (Breidenbach and Beevers, 1967).

Certain yeast species including *Candida tropicalis*, *Candida albicans*, *Yarrowia lipolytica*, *Pichia pastoris* and *S. cerevisiae* are able to assimilate n-alkanes and/or fatty acids via peroxisomal (glyoxysomal) ß-oxidation and glyoxylate pathways (Kunau et al., 1987,1988; Veenhuis et al., 1987). In addition, the glyoxylate cycle aids in the ability of fungi to grow on ethanol or acetate as carbon sources. Ethanol is oxidized to acetaldehyde, then to acetate and acetyl-CoA by the cytosolic enzymes alcohol dehydrogenase, acetylaldehyde dehydrogenase and acetyl-CoA synthase, respectively. The acetyl-CoA is then transferred to glyoxysomes where glyoxylate pathway enzymes use it for biomass synthesis (Veenhuis and Harder, 1987). Interestingly, peroxisome-deficient mutants of *Hansenula polymorpha* are able to grow on ethanol, even though isocitrate lyase and malate synthase are mislocalized to the cytosol (Cregg et al., 1990; Sulter et al., 1991).

1.3.3 Sterol metabolism

In animal cells, peroxisomes contain several enzymes involved in cholesterol biosynthesis including acetoacetyl-CoA thiolase, 3-hydroxy-3-methylglutaryl coenzyme-A reductase, mevalonate kinase and sterol-carrier protein 2 (Krisans, 1992). However, activities of some of these enzymes are also present in the endoplasmic reticulum (ER),

cytosol and mitochondrion; therefore, the contribution of peroxisomes to cholesterol levels in the blood is not certain (Keller et al., 1985; Stamellos et al., 1992).

Bile acids are synthesized from cholesterol via enzymes located in the ER, cytosol, mitochondrion and peroxisome (Russell and Setchell, 1992). Among these enzymes, three are present in the peroxisome and are responsible for the terminal steps involved in side-chain oxidation.

Dolichol is an important sterol present in all membranes and influences membrane fluidity, stability and permeability. The pathway for dolichol synthesis is similar to cholesterol except that isoprene units are sequentially added to farnesyl-P_2, a reaction that takes place in the rat liver peroxisome (Appelkvist and Kalen, 1989).

1.3.4 Plasmalogen biosynthesis

Plasmalogens are ether-linked glycerolipids that are present in all membranes and especially abundant in nervous tissue. They are a major component of the myelin sheath that is essential in the propagation of nerve impulses (Horrocks, 1972). In addition, plasmalogens may protect cell membranes by scavenging oxygen radicals (Zoeller et al., 1988). Peroxisomes are the site of the first three enzymes in plasmalogen synthesis. These enzymes are dihydroxyacetone phosphate (DHAP) acyltransferase, alkyl DHAP synthase, and acyl DHAP reductase (Hajra and Bishop, 1982; Hajra et al., 1979; Jones and Hajra, 1977). The remaining plasmalogen pathway enzymes are located in the ER (Declercq et al., 1984).

1.3.5 Purine catabolism

Urate oxidase (uricase) is a peroxisomal matrix enzyme that oxidizes uric acid to allantoin in certain animals, plants, and fungi. Allantoin is the end-product of purine

catabolism in most mammals and reptiles (Tolbert, 1981). However, in humans, uric acid is the end-product due to the lack of urate oxidase. In the root nodules of leguminous plants, fixed nitrogen is used to form xanthine which is metabolized to uric acid. Urate oxidase present in the peroxisomes of uninfected cells then oxidizes uric acid to allantoin which is distributed to other cellular tissues (Hanks et al., 1981).

1.3.6 Oxidation of D-amino acids

D-amino acid oxidases are present in peroxisomes of both mammals and yeasts (Swensen et al., 1982; Zwart et al., 1983a). Since D-amino acids do not appear to be part of normal metabolism, their physiological function is unknown. The D-amino acid oxidases are flavoproteins and catalyze deamination. As a result of the presence of peroxisomal D-amino acid oxidase, certain yeast species such as *C. boidinii* and *H. polymorpha* are able to use D-alanine as a carbon and/or nitrogen source (Sulter et al., 1990; Zwart et al., 1983a).

1.3.7 Photorespiration and glycolate pathway in plants

Photorespiration involves the uptake of oxygen and release of carbon dioxide (CO_2) during photosynthesis, a process that normally fixes CO_2 and releases oxygen. In the early 1920s, Warburg observed that photosynthesis is inhibited by high oxygen levels (Warburg, 1920). Fifty years later, Ogren and Bowes showed that the site of oxygen action is ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco), the enzyme responsible for CO_2 fixation (Ogren and Bowes, 1971). In the presence of oxygen, this enzyme acts as an oxygenase catalyzing the oxidation of ribulose-1,5-bisphosphate (RuBP) to phosphoglycolic acid. The photorespiration reaction is complex and results in a net loss of fixed carbon as CO_2 without ATP generation. In C_3 plants which use RuBP

for CO_2 fixation, photorespiration can contribute to a 30-40% reduction in biomass yield (Tolbert, 1981).

In 1968, Tolbert and co-workers discovered the glycolate pathway in leaf peroxisomes (Tolbert et al., 1968). This pathway acts as a scavenging mechanism that rescues carbon which would otherwise be lost as glycolate by photorespiration. Phosphoglycolic acid formed by oxidation of RuBP is converted to glycolic acid in the chloroplast and is then translocated to the leaf peroxisome. There it is oxidized to glyoxylate by glycolate oxidase and then transaminated via an aminotransferase to glycine along with the liberation of oxygen. Glycine molecules are then transported to the mitochondrion, where they undergo oxidative decarboxylation catalyzed by glycine decarboxylase to yield CO_2 and NH_3. The methylene group (i.e., formaldehyde) remaining from this glycine is accepted by tetrahydrofolate. The methylenetetrahydrofolate formed then donates its methylene group to a second molecule of glycine to form serine in a reaction catalyzed by hydroxymethyltransferase. Thus for every two molecules of glycine generated from photorespiration, one molecule each of CO_2, NH_3, and serine is formed (Lorimer, 1981; Tolbert, 1981).

Serine is exported from the mitochondria to the peroxisomes, where it undergoes transamination to produce hydroxypyruvate followed by reduction to glycerate by hydroxypyruvate reductase. Glycerate then leaves the peroxisome and enters the chloroplast, where it is phosphorylated to form 3-phosphoglycerate and enters the Calvin cycle. It has been speculated that photorespiration and the glycolate pathway may play an important role in the intracellular transport and interconversion of carbohydrates and nitrogenous compounds (Lorimer, 1981; Tolbert, 1981).

1.3.8 Glycolysis in trypanosomes

In most eukaryotic cells, glycolysis occurs in the cytosol. However, in the bloodstream form of trypanosomes, the first seven enzymes of the glycolytic pathway and two glycerol-metabolizing enzymes are localized in a modified microbody termed the glycosome (Opperdoes and Borst, 1977). This unique compartmentation contributes to the high rate of glycolysis in these parasites which rely solely on glycolysis for energy (Opperdoes, 1987).

1.3.9 Methanol metabolism in yeasts

Methylotrophic yeasts such as *Pichia pastoris*, *Hansenula polymorpha* and *Candida boidinii* are able to utilize methanol as a carbon and energy source (see pathway in Figure 1.1) (Hazeu et al., 1972; Oki et al., 1972). Methanol metabolism begins with its oxidation to formaldehyde by alcohol oxidase, a typical H_2O_2-generating peroxisomal oxidase. The H_2O_2 is then decomposed to water and oxygen in the peroxisome by catalase. Formaldehyde can either enter an assimilatory pathway to produce cell mass or a dissimilatory pathway to generate energy. For the assimilatory pathway, a third peroxisomal enzyme, dihydroxyacetone synthase, condenses formaldehyde with xylulose-5-phosphate to form glyceraldehyde phosphate and dihydroxyacetone phosphate, which then leave the peroxisome and enter a cyclic pathway that generates one molecule of glyceraldehyde phosphate for every three turns of the cycle. For the dissimilatory pathway, formaldehyde is oxidized to formic acid and then carbon dioxide via two cytosolic dehydrogenases. Both dehyrogenase reactions generate NADH which is used by the cell's oxidative phosphorylation system to capture energy (Douma et al., 1985; Veenhuis et al., 1992). Because alcohol oxidase is catalytically inefficient, the cell produces large amounts of the enzyme to compensate. As a result, peroxisomes proliferate enormously and can occupy up to 80% of the total cell volume (Veenhuis and Harder, 1991).

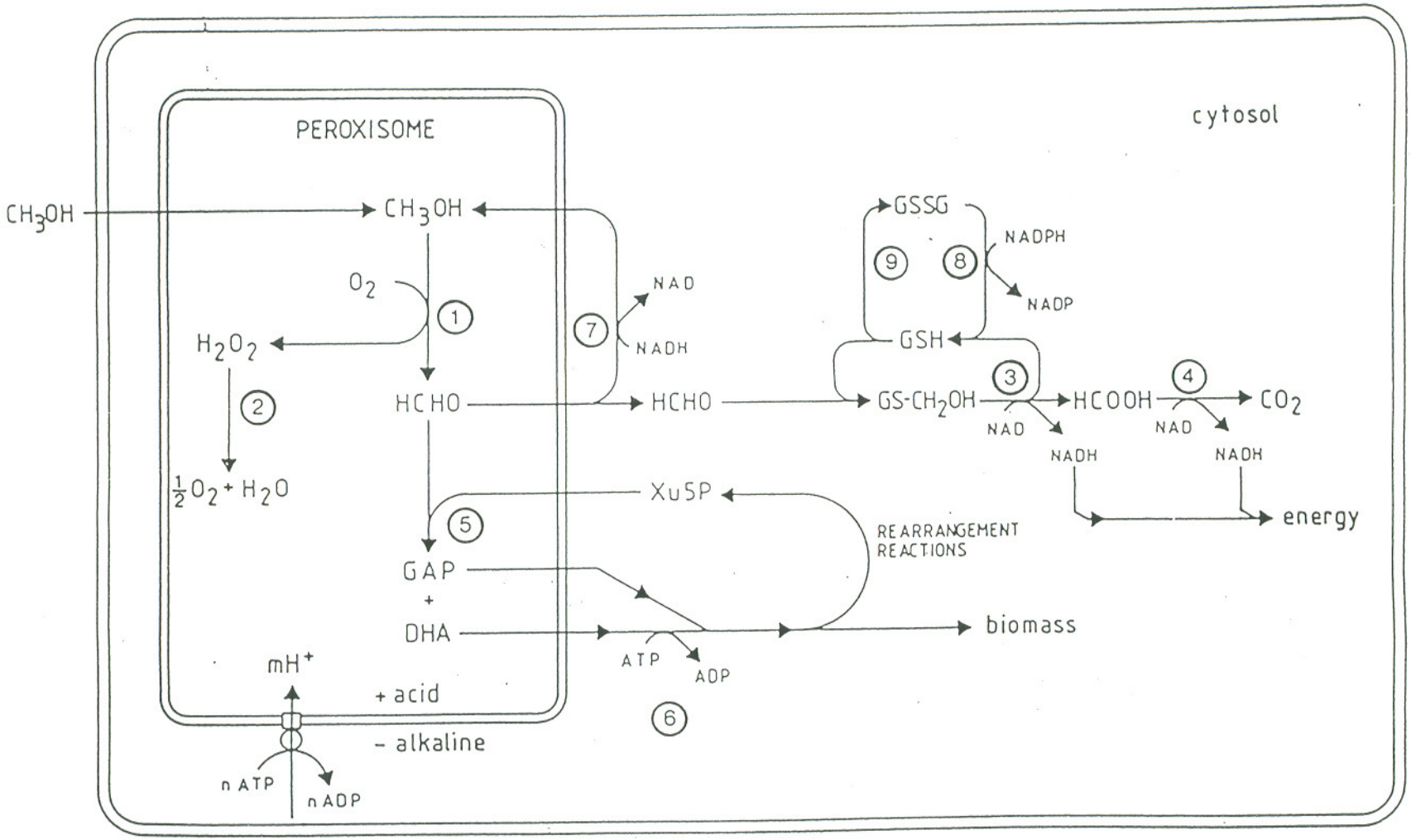

Figure 1.1 Methanol metabolism in wild-type cells of the methylotrophic yeasts.
(1) alcohol oxidase; (2) catalase; (3) formaldehyde dehydrogenase; (4) formate dehydrogenase; (5) dihydroxyacetone dihydroxyacetone kinase; (7) formaldehyde reductase; (8) glutathione reductase; (9) oxidation of glutathione.

1.3.10 Amine metabolism in fungi

Certain fungi can utilize alkylated amines as a nitrogen sources, carbon sources or both (Zwart et al., 1980;1983b). *H. polymorpha* uses methylamine as a nitrogen source via a peroxisomal amine oxidase which oxidizes the substrate to ammonia, formaldehyde and H_2O_2 (Zwart et al., 1983b). The resulting ammonia is assimilated via a peroxisomal glutamate-oxaloacetate aminotransferase with aspartate as the intermediate (Veenhuis and Harder, 1987).

1.4 Human Diseases Affecting the Peroxisome

As might be expected of an organelle with such a central role in metabolism, many genetic diseases exist that affect peroxisomes. These diseases can be divided into three groups based upon the number of peroxisomal enzyme deficiencies associated with each disorder.

1.4.1 Single peroxisomal enzyme deficiencies

The first group is composed of disorders in which a single peroxisomal enzyme is deficient (Lazarow and Moser, 1989; van den Bosch et al., 1992; Subramani, 1993). At least 10 such diseases have been identified (Palosaari et al., 1992; Brown et al., 1993). Three of the better known of these include: X-linked adrenoleukodystrophy (X-ALD; the subject of the film "Lorenzo's Oil") (Rosen, 1993; Valle and Gärtner, 1993), hyperoxaluria type I, and amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease) (Danpure and Jennings, 1986; McNamara and Fridovich, 1993). X-ALD patients suffer the effects of the accumulation of very long chain fatty acids (VLCFAs, C_{24} and greater) due to an inability of a component of the peroxisomal ß-oxidation

system to degrade them (Moser and Moser, 1989). For reasons that are not known, in males this results in a rapid and irreversible deterioration of the myelin sheath surrounding nerve cells and the eventual loss of both voluntary and involuntary motor control and death in boys before age 10. It was thought that the biochemical defect was in peroxisomal VLCFA-CoA synthetase (Wanders et al., 1988; Valle and Gärtner, 1993). Recently, the affected gene was identified and the sequence suggests that its product is a membrane transport protein (Mosser et al., 1993).

Hyperoxaluria type I patients suffer from a deficiency in the peroxisomal enzyme alanine:glyoxylate aminotransferase (AGT) which converts glyoxylate to glycine (Danpure and Jennings, 1986). As a result, excessive amounts of glyoxylate, oxalate and glycolate accumulate in urine. In some patients, the mutation in the AGT introduces an amino acid change that activates a cryptic mitochondrial targeting signal and causes the active enzyme to be mislocalized to the mitochondrion (Purdue et al., 1990, 1991; Purdue and Lazarow, 1994).

ALS was recently shown to be the result of mutations in the Cu/Zn superoxide dismutase which is a peroxisomal enzyme in humans (Rosen et al., 1993). It catalyzes the dismutation of superoxide radicals to oxygen and hydrogen peroxide, thus protecting tissues from oxidative damage by these radicals (McNamara and Fridovich, 1993). It is not clear how a high level of superoxide anions in patients leads to the observed destruction of motor neurons in ALS.

1.4.2 Multiple peroxisomal deficiencies

The second group of peroxisomal disorders is characterized by the loss of some but not all peroxisomal functions (Palosaari et al., 1992; van den Bosch et al., 1992; Subramani, 1993). Examples include the rhizomelic type of chondrodysplasia punctata (RCDP) and Zellweger-like syndrome. Both diseases are similar in that patients lack the

plasmalogen biosynthetic enzyme DHAP acyltransferase, and as a result have reduced levels of plasmalogens (Lazarow and Moser, 1989). In addition, peroxisomal 3-ketoacyl-CoA thiolase is either located in the cytosol or absent, with the result that VLCFAs accumulate in patients (Suzuki et al., 1988). Recent evidence suggests that either or both of these disorders may be the result of a defect in a minor import pathway used by thiolase and a few other peroxisomal enzymes. Thus, those peroxisomal metabolic pathways that require an enzyme imported by this mechanism are deficient while other pathways that do not involve one of these enzymes continue to function normally (Motley et al., 1994).

1.4.3 Generalized impairment in peroxisomal function

The third group of diseases is characterized by a general deficiency in all peroxisomal functions (Lazarow and Moser, 1989; Palosaari et al., 1992; van den Bosch et al., 1992; Subramani, 1993). This group includes Zellweger syndrome (ZS), and three disorders that appear to be milder versions of ZS: neonatal adrenoleukodystrophy, infantile Refsum disease, and hyperpipecolic acidaemia. In ZS patient cells, morphologically recognizable peroxisomes are absent (Santos et al., 1988b) and most peroxisomal matrix enzymes are either located in the cytosol (e.g., catalase, D-amino acid oxidase and L-α-hydroxyacid oxidase) or absent (e.g., DHAP acyltransferase, alkyl-DHAP synthase, all VLCFA ß-oxidation pathway enzymes, pipecolic acid oxidase) (Danks et al., 1975; Schutgens et al., 1984; Wanders et al., 1984; Schrakamp et al., 1985). ZS patients usually die within their first year and there is no known therapy (Wilson et al., 1986).

Cell fusion studies with ZS cell lines indicate that defects in any one of at least 11 different genes can cause ZS (Shimozawa et al., 1993; Subramani, 1993). Although peroxisomes are absent in ZS cells, abnormal vesicular structures termed peroxisomal

ghosts are observed (Santos et al., 1988b). The ghost vesicles contain peroxisomal membrane proteins but not matrix proteins, which suggests that ZS may be the result of defects in the machinery for import of matrix proteins (Santos et al., 1988b). This hypothesis is supported by experiments in which peroxisomal matrix enzymes were microinjected into ZS cells. ZS cells were unable to import microinjected matrix enzymes whereas normal control cells could (Walton et al., 1992a). Recently, it was shown that one group of ZS patients have mutations in a gene encoding a matrix protein import receptor (Dodt et al., 1995) (See Section 1.5.1.2 for further discussion).

1.5 Peroxisome Biogenesis

1.5.1 Import of proteins into peroxisomes

1.5.1.1 Targeting signals

Evidence suggests that at least three different targeting signals are involved in the import of peroxisomal proteins (Zhang et al., 1993b; Purdue and Lazarow, 1994). The first peroxisomal targeting signal (PTS1) was discovered by Subramani and colleagues on firefly luciferase and is composed of a tripeptide sequence, (SKL and conservative variants) located at the carboxyl terminus of many peroxisomal matrix proteins (Gould et al., 1987; Gould et al., 1988; Gould et al., 1989; Swinkels et al., 1992). Addition of a PTS1 to the COOH-terminus of non-peroxisomal proteins such as bacterial chloramphenicol acetyltransferase (bCAT) is sufficient to target that protein to peroxisomes (Gould et al., 1987, 1989). Furthermore, luciferase is properly imported into peroxisomes when expressed in cells of mammals, plants, insects, trypanosomes and yeasts (Gould et al., 1990; Sommer et al., 1992). Thus, the PTS1 import pathway appears to be evolutionarily conserved (Keller et al., 1991).

The second peroxisomal targeting signal (PTS2) is located at the amino terminus of a small number of peroxisomal matrix proteins. The consensus sequence is R/K-L/I-X5-H/Q-L and is present in thiolases of human, rat, *C. tropicalis, Y. lipolytica*, and *S. cerevisiae,* malate dehydrogenase of watermelon, and *T. brucei* glycosomal aldolase (Clayton, 1985; Bout et al., 1988; Gietl et al., 1990; Hijikata 1990; et al., 1990; Igual et al., 1991; Swinkels et al., 1991; Blattner et al, 1995), *H. polymorpha* amine oxidase, and Per1p (Waterham et al., 1994; Bruinenberg et al., 1989). PTS2 sequences are sometimes but not always cleaved after import of proteins into peroxisomes (Erdmann, 1994; Glover et al., 1994b; Swinkels et al., 1991). Similar to PTS1 sequences, addition of a PTS2 to the amino terminus of non-peroxisomal proteins such as bCAT was sufficient to target the protein to peroxisomes (Osumi et al., 1991; Swinkels et al., 1991). Expression of watermelon malate dehydrogenase in *H. polymorpha* under the control of the alcohol oxidase promoter resulted in efficient targeting of the plant enzyme into the yeast peroxisomes. The enzyme was active but, unlike in plant cells, the presequence was not cleaved (Gietl et al., 1986; van der Klei et al., 1993).

Many peroxisomal proteins do not have either a PTS1 or PTS2 sequence. Thus, at least one other PTS may exist. Evidence suggests that internal sequences are essential for targeting of catalase A of *S. cerevisiae* (Kragler et al., 1993) and acyl-CoA oxidase of rat and *C. tropicalis* (Small et al., 1988; Kamiryo et al., 1989; Miyazawa et al., 1989). Peroxisomal integral membrane proteins may be inserted into the peroxisomal membrane via yet another mechanism. None of the peroxisomal membrane proteins described to date (PAF1p, PMP70, PMP22, Pas3p and PMP47) contain either PTS1 or PTS2 (Kamijo et al., 1990; McCammon et al., 1990; Höhfeld et al., 1991; Tsukamoto et al., 1991; Kamijo et al., 1992; Shimozawa et al., 1992; Kaldi et al., 1993; Moreno et al., 1994). In addition, Zellweger cell lines are defective in the import of most matrix proteins but

appear to import membrane proteins normally (Santos et al., 1988a; Santos et al., 1988b; Wiemer et al., 1989).

1.5.1.2 Receptors for import of peroxisomal proteins

As shown for other organellar import machineries, import of peroxisomal matrix proteins appears to involve receptors; that is, proteins which specifically recognize and bind peroxisomal proteins presumably through PTS sequences. Subramani and coworkers found that peroxisome-deficient *pas8* mutants of *P. pastoris* are defective in the import of several PTS1 enzymes but continue to import the PTS2 enzyme thiolase normally (McCollum et al., 1993). They also showed that the *in vitro* synthesized PAS8 product (Pas8p) binds a peptide ending in the PTS1 sequence
SKL but not another non-SKL peptide (McCollum et al., 1993). In addition, *PAS10*, the *S. cerevisiae* homologue of *PAS8*, was isolated from a DNA library by the two-hybrid system when a peptide that ends in SKL was used as target (Brocard et al., 1994). These results suggest that Pas8p (Pas10p) is the PTS1 receptor.

Pas8p is a 65-kDa polypeptide with 7 copies of a degenerate 34-amino-acid sequence called the tetratricopeptide (TPR) or snap helix motif (Goebl and Yanagida, 1991). Deletion of one TPR results in loss of ability of Pas10p to bind SKL in the two-hybrid system assay, suggesting that the TPR motifs are required for binding of SKL-containing proteins (Brocard et al., 1994). The TPR motif exists in proteins involved in RNA processing, transcription, cell cycle control and mitochondrial protein import (Goebl and Yanagida, 1991). Interestingly, the mitochondrial proteins are Mom72p and Mas70p, the putative mitochondrial import receptors from *Neurospora crassa* and *S. cerevisiae*, respectively (Hase et al., 1983; Söllner et al., 1990). Furthermore, similar to the mitochondrial receptors, Pas8p is located on the cytosolic face of the peroxisome in tight association with peroxisomal membrane (Subramani, 1993).

Recently, Gould and coworkers identified a cDNA encoding the probable human homologue of Pas8p which they termed PXR1 (Dodt et al., 1995). They also discovered that one complementation group of ZS cell lines contains mutations in PXR1, indicating that defects in the PTS1 receptor are responsible for the syndrome in this subset of Zellweger patients (Dodt et al., 1995). This represents the first time a mechanistic explanation has been provided for a Zellweger disease and confirms the long-held assumption that ZS is the consequence of defects in peroxisome biogenesis (Santos et al., 1988b).

A candidate for the PTS2 receptor has recently been identified. *S.. cerevisiae pas7* mutant has normal-appearing peroxisomes that contain PTS1 proteins but not the PTS2-enzyme thiolase which remains in the cytosol (Höhfeld et al., 1992; van der Leij et al., 1992). *PAS7* encodes a 42-kDa protein with five repeats of a motif termed the WD-40 repeat (Marzioch et al., 1994). This motif is an approximately 40-amino-acid sequence with a characteristic tryptophan-aspartate (WD) amino acid pair. The WD-40 motif is present in proteins involved in diverse cellular processes such as signal transduction, mitosis, mRNA splicing, and transcription regulation, but its function is not known (van der Voorn and Ploegh, 1992; Neer et al., 1994). In *S. cerevisiae* strains expressing thiolase, Pas7p is found associated with peroxisomes. However, in a thiolase-deficient strain, Pas7p is mostly in the cytosol. These results suggest that Pas7p may cycle between the cytosol and peroxisomes, perhaps bringing a newly synthesized PTS2-protein to the organelle with each cycle (Marzioch et al., 1994).

1.5.1.3 Other Aspects of Protein Import

Aside from the two PTSs and their putative receptors, little is known about peroxisomal protein import mechanism(s). It had been assumed that peroxisomal matrix

proteins are imported into the organelle in an unfolded monomer state and then folded and assembled into active molecules after import into the peroxisome, a model similar to that proposed for translocation and assembly of secretory and mitochondrial proteins. However, recent evidence suggests that many, if not all, peroxisomal proteins assemble in the cytosol and are translocated into peroxisomes as large preassembled multimers (Glover et al., 1994a; McNew and Goodman, 1994). In *S. cerevisiae,* coexpression of bCAT and a construct of bCAT ending in the PTS1 AKL results in import of both bCAT polypeptides into peroxisomes. bCAT without AKL expressed by itself is not imported (McNew and Goodman, 1994). The same results were also observed when the bCAT constructs were expressed in animal cells. Similarly, coexpression of two *S. cerevisiae* thiolases, one with and the other without a PTS, resulted in the efficient import of both (Glover et al., 1994a). The ability of non PTS-containing enzymes to be imported when coexpressed with its PTS-containing counterpart suggests that they form heteromers outside the peroxisome and are imported together.

1.5.2 Genes required for peroxisome biogenesis

A number of genes essential for peroxisome biogenesis have been cloned from humans, rats, Chinese hamster ovary cells and several yeast species. However, with the exception of *PAS8* and *PAS7* (and homologues), a specific role for their products in biogenesis has not been established. Nevertheless, numerous insights into their functions have been obtained through: (1) investigations of mutants defective in the genes; (2) analysis of the primary sequences of their products; and (3) studies of the location and biochemical characteristics of their products. A list of these genes and the characteristics of their products is presented in Table 1.1.

Table 1.1 Genes essential for peroxisome biogenesis.

Pichia pastoris	Hansenula polymorpha	Yarrowia lipolytica	Saccharomyces cerevisiae	Mammals	Location	Structural motifs	References
PAS1 (129)	*PER4* (120)		*PAS1* (117)		Unknown	Hydrophilic, ATP-binding sites (Walker motif)	[1][2][3]
PAS5 (127)		*PAY4* (112)	*PAS8* (116)			Same	[4][5][6]
		PAY2 (42)			Integral membrane protein	Hydrophobic, 2 trans-membrane domains	[7]
	PER8 (34)					Zinc-finger motif	[8]
				PMP70 (70)		ABC transporter motif	[9][10]
				PAF-1 (35)		Zinc-finger motif	[11][12]
	PER9 (52)		*PAS3* (51)			1 transmembrane domain 1 hydrophobic region	[13][14]
PAS8 (65)	*PER3* (68)		*PAS10* (69)	*PXR1* (67)	Peripheral membrane protein, facing cytosol	TPR motif	[15][16] [17][18]
			PAS7 (42)			Hydrophilic, WD-40 motif	[19]
PAS4 (24)			*PAS2* (21)			Ubiquitin-conjugating enzyme	[20][21]

(Continued)

Table 1.1, continued:

Pichia pastoris	Hansenula polymorpha	Yarrowia lipolytica	Saccharomyces cerevisiae	Mammals	Location	Structural motifs	References
PER3 (81)	*PER1* (74)				Peripheral membrane protein facing matrix	AKL at C-terminus	[22][23]
			PAS4 (39)		Unknown	Zinc-finger motif	[24]
			PAS5 (31)		Unknown	Zinc-finger motif	[24]
	PER6 (65)				Unknown	PTS-2 motif	[25]

Molecular masses are given in parentheses.

[1] Heyman *et al.*,1994
[2] Hilbrands, pers.comm.
[3] Erdmann, *et al.*,1989
[4] Spong,Subramani 1993
[5] Nuttley *et al.*,1993
[6] Voorn-Brouwer *et al.*,1993
[7] Eitzen *et al.*,1993
[8] Tan *et al.*,1995
[9] Kamijo *et al.*,1990
[10]Kamijo *et al.*,1992
[11] Tsukamoto *et al.*,1991
[12] Shimozawa *et al.*,1992
[13] Baerends, pers.comm.
[14] Höhfeld *et al.*,1991
[15] McCollum *et al.*,1993
[16] van der Klei *et al.*, subm.
[17] van der Leij *et al.*,1993
[18] Dodt *et al.*,1995
[19} Marzioch *et al.*,1994
[20] Crane *et al.*,1994
[21] Wiebel *et al.*,1991
[22] Liu *et al.*,1995
[23] Waterham *et al.*,1994
[24] Kunau *et al.*,1993
[25] Tuijl *et al.*, unpublished results.

1.5.2.1 Peroxisome proliferation

In *H. polymorpha*, overexpression of Per8p results in a marked increase in peroxisome proliferation over that seen in wild-type cells. This suggests that Per8p may be a component of the peroxisome proliferation machinery (Tan et al., 1995b). In *S. cerevisiae*, induced cells of a *PMP27* deletion strain contain only a few peroxisomes that are much larger than those observed in wild-type cells (Erdmann and Blobel, 1995). This is the phenotype expected of a mutant defective in peroxisome proliferation and suggests that PMP27 also plays a role in this process.

1.5.2.2 A ubiquitin-conjugating enzyme

Pas4p of *P. pastoris* (Crane et al., 1994) and its *S. cerevisiae* homologue Pas2p (Wiebel and Kunau, 1992) display strong similarity to members of the ubiquitin-conjugating enzyme family (Jentsch, 1992). Pas4p contains ubiquitin both *in vitro* and *in vivo* (Crane et al., 1994). Furthermore, a change of the cysteine (thought to be the ubiquitin attachment site) to alanine abolished the function of Pas4p and ubiquitin binding (Crane et al., 1994). The role of ubiquitin conjugation in peroxisome biogenesis is not clear.

1.5.2.3 Transport proteins

PMP70 from a rat cDNA library encodes a protein of 75 kDa (Kamijo et al., 1990). Pmp70 is a peroxisomal integral membrane protein with its hydrophilic carboxyl terminus exposed to the cytosol (Kamijo et al., 1990). The carboxyl terminus has sequence similarity to the ATP-Binding Cassette (ABC) transporter protein family (Higgins, 1992). It has been suggested that PMP70 may be involved in the transport of ions or proteins across the peroxisome membrane (Kamijo et al., 1990). The human

homologue of *PMP70* has also been cloned and is 95% identical to the rat *PMP70* (Kamijo et al., 1992). Mutations in *PMP70* have been found in two Zellweger patients. One is a RNA donor splice site mutation and the other is a missense mutation that substitutes glycine for aspartic acid at amino acid 17 (Gärtner et al., 1992).

PMP47 from the yeast *C. boidinii* encodes a 47-kDa protein that is a peroxisomal integral membrane protein induced by either methanol, oleic acid or D-alanine (Goodman et al., 1990; McCammon et al. 1990). Sequence analysis indicates that it has six-membrane spanning domains and that it belongs to a family of carrier proteins (McCammon et al., 1990; Jank et al., 1993; McCammon et al., 1994; Moreno et al., 1994). The PMP47 PTS is located between amino acids 199 and 267 (McCammon et al., 1994). It is hypothesized that PMP47 is a transport protein for moving substrates from the cytosol to the peroxisome.

1.5.2.4 ATP-binding proteins

At least two genes required for peroxisome biogenesis encode proteins with ATP-binding motifs sometimes referred to as Walker or AAA-protein (ATPases associated with diverse cellular activities) motifs (Kunau et al., 1993). The motif is found twice in *P. pastoris* Pas1p (*S. cerevisiae* Pas1p) and *P. pastoris* Pas5p (*S. cerevisiae* Pas8p; *Y. lipolytica* Pay4p) (Erdmann et al., 1991; Spong and Subramani, 1993; Voorn-Brouwer et al., 1993; Heyman et al., 1994; Nuttley et al., 1994) and is also found in numerous other non-peroxisomal proteins such those involved in vesicle fusion events (Sec18p), cell cycle control (Cdc48p), and transcriptional regulation (TBP-1) (Eakle et al., 1988; Nelbock et al., 1990; Fröhlich et al., 1991). In the *S. cerevisiae PAS1* gene, mutagenesis of a conserved lysine to a glutamine in the second Walker motif results in a loss of Pas1p function. Thus, ATP-binding (and probably hydrolysis) in Pas1p is essential for

peroxisome biogenesis (Krause et al., 1994). All of the peroxisomal ATP-binding proteins are large (greater than 100 kDa) but present at relatively low levels in induced cells (Erdmann et al., 1991; Heyman et al., 1994). At present, their subcellular location(s) and function(s) in peroxisome biogenesis are not known.

1.5.2.5 Cysteine-rich proteins

Several peroxisome biogenesis proteins are characterized by the presence of a C3HC4 motif, a subgroup within the cysteine-rich zinc finger-like domain family (Coleman, 1992; Freemont et al., 1992). Like the ATP-binding motif, the C3HC4 motif is found in a variety of nonperoxisomal proteins, including those involved in DNA repair, transcriptional regulation, vacuolar sorting and vesicle formation/fusion (Jones et al., 1988; Dulic and Riezman, 1989; Kakizuka et al., 1991; Robinson et al., 1991). Zinc fingers are involved in DNA binding (Tagawa et al., 1990). However, the motif exists on several proteins that are not located in the nucleus. As an alternative function for these proteins, it is hypothesized that the motif may also function in protein-protein interactions (Freemont et al., 1992; Kunau et al., 1993).

Peroxisomal biogenesis proteins with this motif include the rat and human PAF1 proteins, the *H. polymorpha* Per8p (and its homologue *S. cerevisiae* Pas4p) (Tsukamoto et al., 1991; Kunau et al., 1993; Tan et al., 1995b). PAF1p is a 35-kDa peroxisomal integral membrane protein. Homozygous mutations in *PAF1* are responsible for one Zellweger syndrome group (Shimozawa et al., 1992). Peroxisome function is restored in cells from these patients when transfected with the wild-type rat or human *PAF1* genes. However, the function of PAF1p in peroxisome biogenesis is not known. As described above, *H. polymorpha* Per8p appears to be a component of the peroxisome proliferation machinery.

1.5.2.6 Peroxisomal targeting signal-containing proteins

The *H. polymorpha* PER1 protein is a 74-kDa polypeptide that contains both a carboxy-terminal PTS1 (AKL) motif and an amino terminal PTS2 motif (Waterham et al., 1994). Either motifs when appended to *E. coli* ß-lactamase, targets this nonperoxisomal protein to peroxisomes. As expected for a PTS-containing protein, Per1p is located in the peroxisomal matrix. It is the only matrix protein known to be required for peroxisome biogenesis (Waterham et al., 1994). As described in this thesis, *P. pastoris* Per3p may be a homologue of Per1p.

1.6 Approaches to the Study of Peroxisome Biogenesis

1.6.1 Biochemical approaches

1.6.1.1 *In vitro* import

In vitro import was one of the first approaches to investigate a specific aspect of peroxisome biogenesis, the translocation of proteins into the organelle. The technique involves the isolation of intact organelles and the subsequent addition of an *in vitro* synthesized and labeled protein to the purified organelles and has provided important information on the protein import machinery of the chloroplasts, mitochondria, ER and other components of the secretory pathway apparatus. For peroxisomal *in vitro* import studies, an increased resistance of the labeled protein to digestion by a protease is taken as evidence that the protein has been imported; i.e., it is resistant because it is within the peroxisome. Peroxisomal import studies of this sort have been performed with rat liver peroxisomes by using labeled rat acyl-CoA oxidase, PMP22, catalase, thiolase and urate oxidase (Fujiki and Lazarow, 1985; Imanaka et al., 1987; Fujiki et al., 1989; Miyazawa et al., 1989; Diestelkötter and Just, 1993; Miura et al., 1994a,1994b). With plant glyoxysomes, *in vitro* import has been examined using labeled malate synthase and

malate dehydrogenase (Kruse et al., 1981;Gietl and Hock, 1984; Gietl and Hock, 1986). In yeasts, import of labeled *S. cerevisiae* catalase into *S. cerevisiae* peroxisomes and labeled *C. tropicalis* acyl-CoA oxidase into *C. tropicalis* peroxisomes have been investigated (Small and Lazarow, 1987; Small et al., 1988; Thieringer et al., 1991). Results of these studies suggest that import of peroxisomal matrix proteins is dependent on time, temperature, ATP and a PTS, but does not require a membrane potential (Imanaka et al., 1987). Interestingly, *in vitro* import of the peroxisomal integral membrane protein PMP22 did not require ATP (Diestelkötter and Just, 1993), a result consistent with the notion that peroxisomal membrane proteins are imported via a different mechanism than that used for matrix proteins (Lazarow, 1989; Aitchison et al., 1992; Lazarow, 1993; Subramani, 1993).

Unfortunately the general use of *in vitro* import for peroxisome studies has been hampered by several limitations of the assay. First, import into peroxisomes is inefficient relative to other systems. Second, no easily observable change occurs upon import of a peroxisomal protein, such as the signal sequence cleavage that take place upon import of proteins into the ER, mitochondrion or chloroplast. Thus, import has been measured solely by sensitivity to protease, an assay that is subject to many artifacts. Third, peroxisomes are very fragile organelles that are difficult to isolate intact and readily leak small molecules and even proteins (Kunau et al., 1993; Subramani, 1993). Finally, in some instances results of *in vitro* experiments have not agreed with those of *in vivo* studies.

1.6.1.2 *In vivo* import

In part due to problems encountered with *in vitro* import, several *in vivo* or semi-*in vivo* techniques were developed to investigate protein import into peroxisomes. One *in vivo* import method is to express peroxisomal proteins in a heterologous host. The host

that is selected is one that does not contain endogenous activity for the foreign protein or in which endogenous proteins do not cross react with antibodies against the foreign protein, so that the fate of the foreign protein can be readily monitored. This general technique was first applied by Subramani and coworkers to identify and define the PTS1 sequence of firefly luciferase in CV1 cells and subsequently been used to define PTSs on a variety of peroxisomal proteins from mammalian cell lines, yeasts, plants and trypanosomes as hosts (Gould et al., 1987, 1989, 1990; Swinkels et al., 1991; Sommer et al., 1992). More recently, the PTS2 of watermelon malate dehydrogenase was identified by expression of its gene (and mutant variants) in the yeast *H. polymorpha* (van der Klei et al., 1993; Gietl et al., 1994). Interestingly, the presequence containing PTS2 is cleaved after import into plant peroxisomes but not in this yeast (Gietl et al., 1986; van der Klei et al., 1993). As a second example, *C. tropicalis* acyl-CoA oxidase and hydratase-dehydrogenase-epimerase are targeted into peroxisomes of *C. maltosa* and *C. albicans*, respectively; this indicates that these enzymes contain internal PTSs (Kamiryo et al., 1989; Aitchison and Rachubinski, 1990). Unfortunately, the heterologous expression method is mainly limited to defining PTSs and is not useful in investigations of other aspects of import.

Microinjection of peroxisomal enzymes into mammalian cell lines is a second *in vivo* method that has shown promise (Walton et al., 1992a, 1992b). Proteins injected into cells are visualized by indirect immunofluorescence and peroxisomal import is judged to have occurred if the injected protein co-localizes in punctate structures with a known endogenous peroxisomal matrix enzyme such as catalase. Import was shown to be time-, temperature- and PTS-dependent (Walton et al., 1992a). Surprisingly, it was discovered that human serum albumin (HSA) modified by random cross-linkage of peptides ending in SKL is imported into peroxisomes. This result suggested that import does not

necessarily require an unfolded peptide with a PTS at its terminus but may occur with prefolded proteins. It was also shown that certain Zellweger cell lines could not import luciferase or HSA modified with SKL peptides (Walton et al., 1992b) indicating that microinjection results can accurately reflect *in vivo* events and supporting the hypothesis that these cell lines are defective in a component of their PTS1 import pathway (Santos et al., 1988b).

A third assay method involves permeabilizing the plasma membrane of tissue-culture cells by using the bacterial toxin streptolysin-O (Rapp et al., 1993; Wenderland and Subramani, 1993). The concentration and timing of treatment is controlled so as not to disrupt peroxisomal membranes. Results of these studies are consistent with *in vitro* results, in that import is PTS-, time- and temperature-dependent, and requires ATP hydrolysis but not a membrane potential (Rapp et al., 1993; Wenderland and Subramani, 1993). Interestingly, import requires an N-ethylmaleimide-sensitive membrane factor(s) as well as a cytosolic SKL-binding factor and a 70-kDa-class heat shock protein (Wenderland and Subramani, 1993; Walton et al., 1994).

1.6.2 Molecular genetic approaches

1.6.2.1 Forward genetic approach

Two general molecular genetic approaches have been applied to study peroxisome biogenesis (Kunau et al., 1993). One is essentially a forward genetic approach that requires the isolation of mutants with defects in peroxisome biogenesis. Wild-type alleles of the defective peroxisomal genes are then isolated from a DNA library by functional complementation, (i.e., the ability of the wild-type peroxisomal gene to restore normal peroxisomes and a wild-type phenotype to a peroxisome-deficient mutant strain). The characteristics of mutants and the products of these genes are then

studied to generate insights into their function in the biogenesis process.

The forward genetic approach has been most successfully exploited in yeasts where the necessary mutants in peroxisome biogenesis (peroxisome-deficient mutants) are readily isolated. Peroxisome-deficient yeast strains are ideal conditionally lethal mutants in that they are healthy in glucose medium but nonviable only when cultured on a carbon source that requires peroxisomes for metabolism (e.g., methanol and/or oleate) (Erdmann et al., 1989; Cregg et al., 1990; Gould et al., 1992; Liu et al., 1992; Waterham et al., 1992; Nuttley et al., 1993). To date, peroxisome-deficient mutants affected in 15 genes have been described in *S. cerevisiae* (Erdmann and Kunau, 1992; van der Leij et al., 1992; Elgersma et al., 1993; Kunau et al., 1993), 14 genes in *H. polymorpha* (Cregg et al., 1990; Waterham et al., 1992; Tan et al., 1995a), 10 genes in *P. pastoris* (Gould et al., 1992; Liu et al., 1992; Subramani, 1993), and two genes in *Y. lipolytica* (Nuttley et al., 1993). In all these peroxisome-deficient yeast mutants, most peroxisomal matrix enzymes are synthesized but are mislocalized to the cytosol.

Most of the yeast peroxisome-deficient mutants were isolated by screening mutagenized cultures for strains that were unable to grow on methanol (*H. polymorpha*) (Cregg et al., 1990; Waterham et al., 1993; Tan et al., 1995a), oleate (*S. cerevisiae* and *Y. lipolytica*) (Erdmann et al., 1989; Kunau and Hartig, 1992; Elgersma et al., 1993; Nuttley et al., 1993; Zhang et al., 1993, 1993b) or both (*P. pastoris*) (Gould et al., 1992; Liu et al., 1992) but still capable of growth on other carbon sources. Recently, a rapid positive selection scheme for isolating peroxisome-deficient *S. cerevisiae* mutants was described (Elgersma et al., 1993). In *S. cerevisiae,* expression of the bleomycin resistance gene confers resistance to the antifungal drug phleomycin. A chimeric bleomycin-resistance protein fused to the carboxy-terminus of luciferase is imported into peroxisomes where it cannot efficiently detoxify phleomycin. Thus, strains expressing

the fusion are sensitive to the drug. Selection for strains that are resistant to phleomycin strongly enriches for peroxisome-deficient mutants since such mutants no longer import the bleomycin resistance-luciferase fusion protein and the now cytoplasmic-protein is again free to detoxify the drug.

Although more difficult to apply to higher eukaryotes, the forward genetic method has been accomplished in at least one instance. Peroxisome-deficient CHO cell lines have been isolated by a colony assay for loss of activity for the plasmalogen biosynthetic enzyme DHAP acyltransferase (Zoeller and Raetz, 1986). One of the resulting CHO cell mutant lines was then utilized to clone the rat peroxisomal biogenesis gene *PAF1* by functional complementation from a rat liver cDNA expression library (Tsukamoto et al., 1991).

1.6.2.2 Reverse genetic approach

The other approach is a reverse genetic strategy that involves the purification of a peroxisomal protein that is thought be required for biogenesis (Kunau et al., 1993). The gene encoding the protein is then cloned through antibodies generated against the protein or through labeled hybridization probes designed from sequenced portions of the protein. The advantage of the forward genetic approach is that it is possible to know with certainty that the genes to be isolated are essential for peroxisome biogenesis whereas this cannot be pre-determined with the reverse genetic approach. However, the reverse genetic approach has the potential to yield peroxisome biogenesis genes that, for various reasons, could not be cloned by the forward genetic approach.

The reverse genetic approach has been used to clone genes encoding a 70-kDa peroxisomal integral membrane protein (*PMP70*) from a rat liver cDNA library (Kamijo et al., 1990) and a 27-kDa peroxisomal integral membrane protein (*PMP27*) from *S.*

cerevisiae (Erdmann and Blobel, 1995). Both of these genes were subsequently shown to be required for peroxisome biogenesis. Other genes cloned by this approach include the *C. boidinii PMP20, PMP31, PMP32* and *PMP47* (Garrard and Goodman, 1989; McCammon et al., 1990; Moreno et al., 1994). However, evidence for a function in peroxisome biogenesis has yet to be demonstrated with these genes.

1.7 Thesis Summary

The methylotrophic yeast *P. pastoris* is an excellent model system to study peroxisome biogenesis. In addition to the general advantages of working with a yeast (e.g., single-celled microorganism with rapid growth rate, small genome size, and predominantly haploid life cycle), *P. pastoris* is capable of growth on methanol or oleate, carbon sources that require multiple peroxisomal enzymes to be metabolized (see section 1.3 for discussion of pathways). Growth on either of these substrates greatly increases both the size and number of the organelles. This facilitates light and electron microscopic observation of peroxisomes as well as the purification of the organelles for biochemical studies. As a result of previous work in this and other laboratories, techniques for classical and molecular-genetic manipulations of *P. pastoris* are well developed (Cregg et al., 1985; Digan and Lair, 1986). Mutants can be readily isolated, backcrossed and organized into complementation groups. *P. pastoris* genes are easily cloned by functional complementation and specific *P. pastoris* mutants can be created by the gene replacement technique (McCollum et al., 1993; Spong and Subramani, 1993; Crane et al., 1994; Heyman et al., 1994; Liu et al., 1995).

This thesis describes the exploitation of *P. pastoris* as a model system for genetic studies of peroxisome biogenesis. In Chapter 2, I describe the isolation and

characterization of *P. pastoris* mutants that are defective in peroxisome biogenesis (*per* mutants). The mutants were isolated by screening a collection of methanol-utilization defective (Mut^-) strains for ones that were also oleate-utilization defective (Out^-). Electron microscopic examination revealed that virtually all of these mutants lacked morphologically recognizable peroxisomes and thus were *per* mutants (Liu et al., 1992). Furthermore, biochemical studies showed that in methanol- and oleate-induced cells of each *per* mutant, the peroxisomal marker enzyme catalase was present and active but located in the cytosol. On the other hand, alcohol oxidase, the first enzyme in the methanol pathway, was present at only very low levels in methanol-induced cells of the *per* mutants (Liu et al., 1992).

In Chapter 3, I describe the cloning of one of the peroxisomal biogenesis genes *PER3* and the characterization of its product Per3p. The DNA sequence of *PER3* predicts that Per3p is a hydrophobic protein of 81 kDa. I show that Per3p is tightly associated with the peroxisomal membrane. Insights into the function of Per3p in peroxisome biogenesis came from detailed studies of the characteristics of *per3* mutants. A chemically induced *per3* mutant was found to harbor abnormal vesicular structures which contained the PTS2-enzyme thiolase but very little of each of several PTS1 enzymes which were located mostly in the cytosol. These vesicles appear to be peroxisomal remnants similar to those observed in human Zellweger cell lines. Furthermore, the fact that this *per3* mutant is primarily defective in the import of PTS1 enzymes suggests that Per3p plays a role in the PTS1 import pathway. In contrast, a mutant in which most of *PER3* had been deleted lacked peroxisomal remnants and was defective in the import of both PTS1 and PTS2 enzymes. I propose that Per3p is part of the peroxisome import machinery and may be composed of independent domains involved in the import of PTS1 and PTS2 proteins (Liu et al., 1995).

CHAPTER 2

AN EFFICIENT SCREEN FOR PEROXISOME-DEFICIENT MUTANTS OF *PICHIA PASTORIS*

2.1 Summary

We describe a rapid and efficient screen for peroxisome-deficient (*per*) mutants in the yeast *Pichia pastoris*. The screen relies on the unusual ability of *P. pastoris* to grow on two carbon sources, methanol and oleic acid, both of which absolutely require peroxisomes to be metabolized. A collection of 280 methanol-utilization-defective (Mut^-) *P. pastoris* mutants was isolated, organized into 46 complementation groups and tested for those that were also oleate-utilization-defective (Out^-) but still capable of growth on ethanol and glucose. Mutants in 10 groups met this phenotypic description, and 8 of these were observed by electron microscopy to be peroxisome deficient (Per^-). In each *per* mutant, Mut^-, Out^- and Per^- phenotypes were tightly linked and therefore were most likely due to a mutation at a single locus. Subcellular fractionation experiments indicated that the peroxisomal marker enzyme catalase was mislocalized to the cytosol in both methanol- and oleate-induced cultures of the mutants. In contrast, alcohol oxidase, a peroxisomal methanol-utilization-pathway enzyme, was virtually absent from *per* mutant cells. The relative ease of *per* mutant isolation in *P. pastoris*, in conjunction with well-developed procedures for its molecular and genetic manipulation, make this organism an attractive system for studies on peroxisome biogenesis.

2.2 Introduction

Virtually all eukaryotic cells harbor single-membrane-bound organelles called peroxisomes. They are the site of hydrogen peroxide-generating oxidative reactions in cells and, almost without exception, contain the heme enzyme catalase to break down this reactive compound (Lazarow and Fujiki, 1985; Borst, 1989). Enzymes found within the peroxisome matrix are involved in a variety of important metabolic pathways. However, the specific pathways vary significantly depending upon the organism. In humans, peroxisomal enzymes are known to play an essential role in a number of anabolic and catabolic pathways, particularly in lipid metabolism (Lazarow and Moser, 1989). The importance of peroxisomes to humans is dramatically demonstrated by a family of lethal genetic disorders called Zellweger syndrome in which peroxisomes appear to be absent from cells (Lazarow and Moser, 1989; Wilson, 1991).

In recent years, basic features of peroxisome biogenesis have emerged. Peroxisomes do not appear to be synthesized *de novo* or to arise by budding from the endoplasmic reticulum (ER) as do secretory-pathway organelles. Instead, peroxisomes form by budding from pre-existing peroxisomes (Veenhuis et al., 1978). Proteins destined for peroxisomal localization are synthesized on cytosolic or free ribosomes and are post-translationally imported (Fujiki et al., 1984). To date, two peroxisomal targeting signals (PTSs) have been defined. A few peroxisomal enzymes, including rat 3-ketoacyl-CoA thiolase and watermelon malate dehydrogenase, are directed to the organelles by an amino-terminal signal sequence that is proteolytically cleaved upon import, a feature reminiscent of signal sequences on proteins destined for import into the ER, mitochondrion or chloroplast (Osumi et al., 1991; Swinkels et al., 1991). However, the more commonly utilized PTS is a tripeptide sequence, serine-lysine-leucine (and a few

conservative variants), located at the carboxy-terminus of many peroxisomal proteins (Gould et al., 1987, 1990; Keller et al., 1991). Additional PTS systems may exist as well. As observed for other organelles, peroxisomal protein translocation requires ATP hydrolysis (Imanaka et al., 1987). In addition, a proton gradient exists across the peroxisomal membrane (Nicolay et al., 1987), although its role in protein import, if any, is not clear.

To date, genetic methods have not been extensively utilized to investigate peroxisomes. In fact, the first description of a peroxisomal genetic defect was Zellweger syndrome (Goldfischer et al., 1973). Cell fusion results indicate that the syndrome is a consequence of mutations in any one of at least 6 different genes (Brul et al., 1988; Lazarow and Moser, 1989; Roscher et al., 1989). Although Zellweger cells were once thought to be completely devoid of peroxisomes, recent studies have revealed the presence of single-membrane-bound vesicles containing peroxisomal membrane proteins (Lazarow et al., 1986; Santos et al., 1988a; Santos et al., 1988b). Since these structures, termed peroxisomal "ghosts," are without most matrix enzymes, it appears that the primary defect in Zellweger cells is a general inability to import these enzymes (Santos et al., 1988b; Walton et al., 1992a). Peroxisome-deficient mutants have also been reported in Chinese hamster ovary cell lines (Zoeller and Raetz, 1986; Zoeller et al., 1989; Tsukamoto et al., 1990) and in the yeasts, *Saccharomyces cerevisiae* (Erdmann et al., 1989) and *Hansenula polymorpha* (Cregg et al., 1990; Didion and Roggenkamp, 1990). In *S. cerevisiae*, two peroxisome-deficient mutants were identified among a collection of mutants defective in ability to grow on oleate, a carbon source whose metabolism requires a peroxisomal ß-oxidation system in yeasts. Similarly, two *H. polymorpha* peroxisome-deficient mutants were isolated from a collection of mutants defective in ability to grow on methanol (Cregg et al., 1990).

We are pursuing a genetic approach toward understanding peroxisome biogenesis and have selected the methylotrophic yeast *Pichia pastoris* as a model system. For these studies, *P. pastoris* presents certain advantages. First, peroxisomes are absolutely required for the metabolism of methanol as a consequence of the presence in the peroxisome of three pathway specific enzymes, alcohol oxidase, catalase and dihydroxyacetone synthase (Veenhuis and Harder, 1987). Second, methanol growth is robust and mutants that are defective in its utilization are easily identified through standard replica plate screening methods. Third, peroxisomes in methanol-induced cells become numerous and massive (Veenhuis et al., 1978, Veenhuis and Harder, 1987), a useful feature since the primary means of identifying peroxisome-deficient mutants is by direct electron-microscopic examination. Fourth, *P. pastoris* also grows on oleic acid (Subramani and Gould, 1992). Thus, *P. pastoris* is unique in that two easily observable phenotypes are available to screen for peroxisome-deficient mutants. Fifth, techniques for both classical- and molecular-genetic manipulation of this organism are highly developed (Cregg et al., 1985; Digan and Lair, 1986; Cregg, 1987; Cregg et al., 1989).

Our general strategy is to first isolate a comprehensive collection of peroxisome-deficient yeast mutants and then utilize the mutants to clone the affected genes by complementation. In this report, we describe an efficient screen for the isolation of peroxisome-deficient mutants (*per* mutants) of *P. pastoris*. We report the identification of *per* mutants defective in 8 different genes and the effect of the mutations on the presence and subcellular localization of selected peroxisomal matrix enzymes.

2.3 Materials and Methods

2.3.1 Strains, media and growth conditions

Pichia pastoris strains used in this study are listed in Table 2.1. Yeast strains were grown in either YPD (1% yeast extract, 2% peptone, 2% glucose) or YNB (0.67% yeast nitrogen base without amino acids) medium supplemented with one of the following carbon sources: 2% glucose, 1% glycerol, 0.5% ethanol, 0.5% methanol, 0.1% oleic acid. Oleic acid medium was additionally supplemented with 0.5% Tween 40 to solubilize oleate. Amino acids were added to 50 mg/ml as required. Sporulation medium contained 0.5% sodium acetate, 1% potassium chloride, 1% glucose. To measure growth of *P. pastoris* strains on oleic acid, cells were precultured in YPD to an OD_{600} of approximately 1.0 and transferred by centrifugation to YNB medium with Tween 40 and oleate at an initial OD_{600} of about 0.1. Since *P. pastoris* grows to a significant extent on Tween 40 alone, parallel cultures of each strain were incubated in YNB medium with Tween 40 but without oleate. Oleate growth was judged as the difference in growth between cultures with and without oleate. To prepare methanol-induced cells, each strain was precultured in YPD medium and shifted by centrifugation into YNB plus methanol medium. Methanol-utilization-defective (Mut^-) strains were inoculated into methanol medium at a starting OD_{600} of 0.5 and harvested at the times indicated in the text. Mut^+ strains were inoculated at an OD_{600} of 0.1 and harvested at an OD_{600} of about 1.0. Oleate-induced cells were prepared in the same manner except for the substitution of YNB medium plus Tween 40 and oleic acid.

2.3.2 Mutant isolation

The procedure for mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was described in Cregg et al. (1990), except that cells were grown at 30°C. Mutagenized cultures were brought to 30% in glycerol and stored frozen at -70°C until used. NTG treatment resulted in the death of approximately 90% of the cells and freezing killed an

Table 2.1 *P. pastoris* strains used

Strain	Genotype	Number of alleles	Source (reference)
JC100	*wild type*		NRRL Y-11430[a] Cregg *et al*. (1985)
GS115	*his4*		NRRL Y-15851 Cregg *et al*. (1985)
GS190	*arg4*		NRRL Y-18014 Cregg, J.M. (1987)
MS105	*fld*[b]		Cregg, J.M. Unpulished data
JC101	*per1*	5	This study
JC105	*per2*	6	This study
JC111	*per3*	1	This study
JC112	*per4*	1	This Study
JC113	*per5*	1	This study
JC114	*per6*	1	This study
JC115	*per7*	3	This study
JC118	*per8*	5	This study
JC126	*out1*		This study

[a]Northern Regional Research Laboratories, Peoria, IL.

[b]Formaldehyde dehydrogenase defective.

additional 90%. To isolate Mut$^-$ mutants, mutagenized frozen cultures were thawed, washed twice in 25 ml sterile water and spread on YNB agar plates with 0.1% glucose at a dilution that produced approximately 500 colonies per plate. After 3 days at 30^{o}C, colonies which formed were replica-plated onto two sets of YNB plates, the first supplemented with methanol and the second with glucose. After 3 days of incubation at 30^{o}C, colonies which failed to grow on methanol were isolated and retested for tightness of Mut$^-$ phenotype, reversion rate and glucose growth rate.

2.3.3 Genetic analysis

Mating and sporulation procedures were modified versions of those previously described (Cregg et al., 1989). For complementation testing of the Mut$^-$ strain collection, each mutant to be tested was patched onto a YPD agar plate (up to 16 strains per plate) and spread onto a second YPD plate at a density sufficient to form a lawn (~1 x 10^7 cells/plate). After incubation at 30^{o}C overnight, each patch plate was paired with a lawn plate, and the plates were serially replica-plated onto sporulation agar medium to initiate mating. Cells were incubated at 30^{o}C for 2 days on sporulation plates and then replica-plated onto YNB agar supplemented with methanol to select for Mut$^+$ complementing diploid cells. After incubation for at least 3 days at 30^{o}C, plates were scored. As a control for reversion, the procedure was also performed in parallel with plates that contained individual unmixed strains.

Procedures for backcrossing and random spore analysis in *P. pastoris* began with mating of desired sets of mutants and the selection of diploids as described for complementation analysis, except that mating was allowed to proceed for only one day at 30^{o}C prior to replica-plating onto an appropriate diploid selection medium. The purpose of the limited mating period was to assure that diploids did not have time to sporulate

prior to transfer to selection medium. Diploid colonies resulting from each mating were transferred onto a fresh plate of selection medium agar, incubated at 30°C overnight and then onto YPD plates followed by another overnight incubation. Diploid colonies were then streaked onto sporulation plates and incubated for 3 to 4 days at 30°C.

Spore analysis procedures were essentially as described in Gleeson and Sudbery (Gleeson and Sudbery, 1988). Material from the spore plates was transferred to 1 ml of sterile water and mixed. To preferentially kill remaining vegetative cells, an equal volume of ethyl ether was added to the spore suspensions which were then mixed vigorously and allowed to stand at room temperature for about 20 minutes. The spore preparations were then serially diluted with water, and aliquots of selected dilutions were spread on YPD plates. After incubation for 2 to 3 days at 30°C, the resulting colonies were picked and streaked onto sets of plates containing agar media appropriate for phenotypic determination.

2.3.4 Preparation of cell-free extracts

To prepare cell-free extracts, 50 OD_{600} units of each culture were washed twice by centrifugation with ice-cold 50 mM potassium phosphate buffer, pH 7.0, resuspended in 400 µl of the same buffer and transferred to a 13 x 100-mm glass tube containing 0.5 g of 0.5 mm-diameter acid-washed glass beads. The mixture was vigorously mixed for 1 minute using a bench-top vortexer and held on ice for at least 1 minute. This cycle of mixing and cooling was repeated a total of 4 times for each sample. Extracts were then transferred to a 1.5-ml micro-centrifuge tube. Extract which remained trapped in the glass beads was recovered by washing the beads with 300 µl of cold buffer and adding this wash to the extract. To remove cell debris, extracts were centrifuged for 5 minutes at approximately 10,000 x g. The top 400 µl of supernatant was then transferred to a fresh micro-centrifuge tube and held on ice for assay.

2.3.5 Cell fractionation

Protoplasts were prepared, lysed and subjected to differential centrifugation by a modification of the procedure described by Kamiryo et al. (1982). Approximately 500 OD_{600} units of induced culture were washed twice with 10 ml of distilled water, once in 10 ml of MOPS buffer plus DTT [5 mM K 3-(N-morpholino) propanesulfonate, pH 7.2, 0.5 M KCl, 10 mM dithiothreitol (DTT)], and once in 10 ml of MOPS buffer without DTT. Cells were then converted to protoplasts by the addition of 0.8 mg of Zymolyase 100T (ICN, Costa Mesa, CA) and incubation at 30°C for 45 minutes. All subsequent steps were performed at 4°C. Protoplasts were collected by centrifugation at 3,000 x g for 8 minutes and osmotically lysed by gentle resuspension in 5 ml of Sorbitol-MES buffer [5 mM K 2-(N-morpholino) ethanesulfonic acid, pH 6.0, 0.5 mM EDTA, 0.6 M sorbitol, 0.1% ethanol]. Resulting mixtures were then homogenized in a Potter-Elvehjem tissue grinder. Samples were centrifuged at 3,000 x g for 10 minutes to remove unbroken cells and other debris. Supernatants were collected and subjected to a second centrifugation at 20,000 x g for 20 minutes. The resulting peroxisome-containing pellets were resuspended in 500 ml of Sorbitol-MES buffer and, along with supernatants, were held on ice until assay.

2.3.6 Miscellaneous methods

Total protein was measured by the method of Bradford using bovine serum albumin (BSA) as standard (Bradford, 1976). Alcohol oxidase (van der Klei et al., 1990), catalase (Ueda et al., 1990), acyl-CoA oxidase (Dommes et al., 1981) and fumarase (Tolbert 1974) activities were measured according to published procedures. Specific activities for alcohol oxidase, acyl-CoA oxidase and fumarase were expressed in units. One unit was defined as one micromole of product per minute per milligram of

protein. Catalase specific activity was expressed as ΔE_{240} per minute per milligram of protein. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis and Western blotting procedures were performed as previously described (Laemmli, 1970; Sambrook et al., 1989). Nitrocellulose filters were incubated with affinity-purified rabbit antibody against alcohol oxidase of *P. pastoris* (a gift from M. Gleeson, SIBIA, La Jolla, CA). Protein-antibody complexes were visualized using alkaline phosphatase-conjugated goat anti-rabbit antibody and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) as substrate (Sambrook et al., 1989). Purified *P. pastoris* alcohol oxidase was purchased from Sigma Chemical Co., St. Louis, MO. Yeast cell sections were prepared for electron microscopy as described in Cregg et al. (1990) or in McConnell et al. (1990).

2.4 Results

2.4.1 Mutant isolation and screening

Cultures of wild-type *P. pastoris* were subjected to NTG mutagenesis, and stored frozen until use. To isolate methanol-utilization-defective (Mut$^-$) mutants, aliquots of mutagenized cultures were thawed, spread on minimal glucose agar and after incubation to allow colony formation, were screened by replica plating for growth on methanol. Approximately 0.5% of cells surviving mutagenesis and freezing were Mut$^-$ and 280 such mutants were collected.

The Mut$^-$ collection was organized into complementation groups. *P. pastoris* is a homothalic haploid yeast and initiates its sexual cycle in response to nitrogen starvation (Digan and Lair, 1986; Cregg et al., 1989). For complementation testing, each Mut$^-$ mutant was mated pairwise with each of the other mutants as described under Materials and Methods, and complementing diploids were selected by growth on methanol medium agar. A few mutants failed to complement any of the other mutants and were not examined further. These mutants may have harbored secondary mutations affecting mating or dominant Mut$^-$ alleles. The complementation results indicated that the collection represented mutations in approximately 46 different genes whose products are required for growth on methanol. From previous studies, the specific genetic defects were known for two groups. One group was defective in *AOX1*, the primary source of alcohol oxidase in *P. pastoris* (Cregg et al., 1989). The other group was defective in *FLD* which encodes formaldehyde dehydrogenase, a cytosolic methanol-pathway enzyme required for energy generation from methanol (Cregg, J.M. Unpublished data).

Representative Mut$^-$ strains from each complementation group were further examined for utilization of oleic acid and ethanol, carbon sources that along with

methanol, are indicators of the state of peroxisomes in yeasts. Oleate metabolism has been shown to require a peroxisomal ß-oxidation system (Veenhuis et al., 1987; Erdmann et al., 1989). In contrast, peroxisomes are not required for growth on ethanol (Erdmann et al., 1989; Cregg et al., 1990; Sulter et al., 1991). Thus, we anticipated that peroxisome-deficient mutants of *P. pastoris* would be phenotypically Mut$^-$ and oleic acid-utilization-defective (Out$^-$) but still ethanol-utilization-proficient (Eut$^+$). Because *P. pastoris* grows much more readily on ethanol than oleate, the Mut$^-$ collection was first screened for growth on ethanol. Of the 46 Mut$^-$ groups, 34 were observed to be Eut$^+$.

Oleate is a relatively poor growth substrate for *P. pastoris*. Therefore, to best quantify growth, mutant strains were cultured in liquid oleate medium and culture densities were determined by spectrophotometry. Particular attention was paid to cell growth attributable to Tween 40 which was present in oleate medium to help solubilize the fatty acid (Erdmann et al., 1989). Typical results for wild-type *P. pastoris* and an oleic acid-utilization-defective (Out$^-$) strain are shown in Figure 2.1. Within 24 hours after shift to medium without oleate, both wild-type and mutant cultures had increased in density to approximately the same extent, the result of growth on Tween 40 and residual YPD. In the presence of oleate, the wild-type culture reached a cell density of approximately four times that of the same culture without oleate. In contrast, Out$^-$ mutants showed no further increase in culture density above that observed in medium without oleate. Representatives of each Mut$^-$ but Eut$^+$ group were examined for oleic acid utilization and 10 groups were found that were both Mut$^-$ and Out$^-$ but Eut$^+$, the phenotype expected of peroxisome-deficient mutants. One representative of each of the 10 groups was crossed 3 to 5 times against *his4* and *arg4* but otherwise wild-type *P. pastoris* strains and the resulting backcrossed strains were analyzed further.

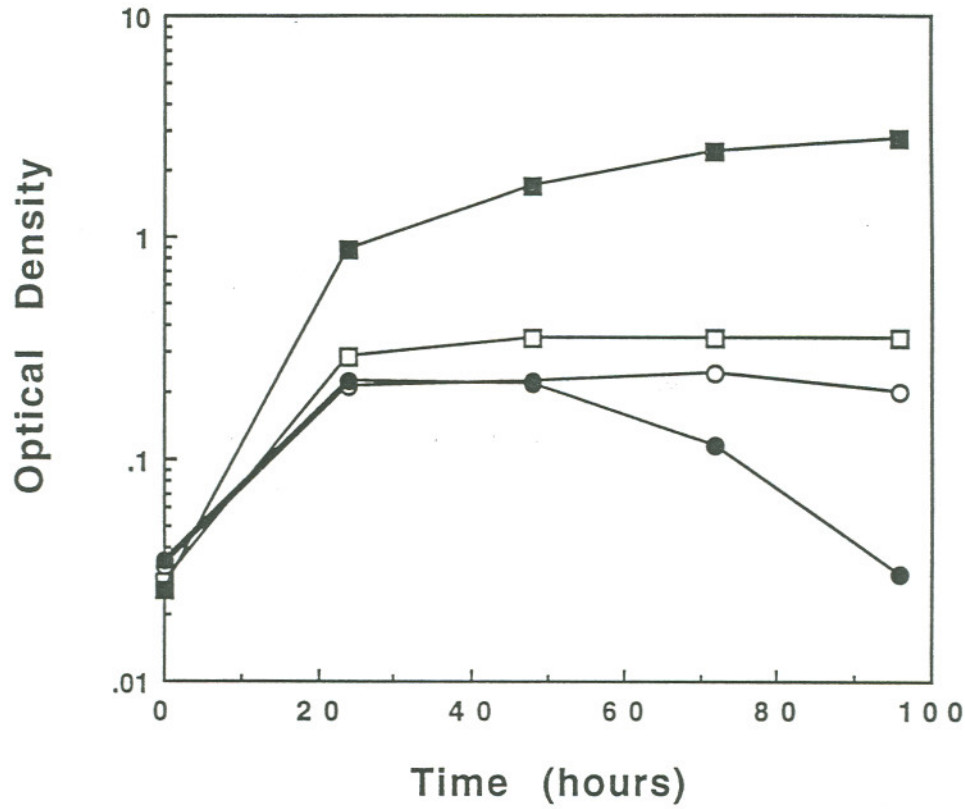

Figure 2.1. Growth of *P. pastoris* on oleic acid. Time 0 is point at which YPD precultures were shifted to oleic acid growth test medium. Strains shown are JC100 (wild-type) in medium with (■) or without oleate (□); JC101 (*per1*) in medium with (●) or without (○) oleate.

2.4.2 Identification of peroxisome-deficient mutants.

The 10 peroxisome-deficient candidates were subjected to extensive electron microscopic (EM) examination for the presence of peroxisomes. In preparation for this, each mutant was precultured in YPD medium and then incubated for approximately 20 hours in medium containing methanol, conditions that lead to an increase in both the size and number peroxisomes in wild-type *P. pastoris*. Under EM examination, most sections of methanol-induced wild-type cells contained readily observable clusters of large peroxisomes (Figure 2.2A). Sections derived from 2 of the 10 mutants contained peroxisomes that were comparable in size, number and morphology to those observed in wild-type *P. pastoris* cells (not shown). The remaining 8 mutants appeared to be peroxisome deficient in that cell sections derived from each were devoid of peroxisomes of the sort normally observed (Figure 2.2B). In 7 of the 8 mutants, a few cell sections were observed that contained small irregularly shaped single-membrane-bound vesicles. (Figure 2.2C and 2.2D). The proportion of sections that displayed these vesicles varied between strains. One of the mutants, JC111, also contained cytosolic proteinaceous aggregates in addition to small membranous vesicles (Figure 2.2E). Finally, the eighth mutant, JC101, appeared to be completely peroxisome deficient in that not even abnormal vesicles were observed. Peroxisome deficiency in these mutants was not an indirect consequence of their inability to metabolize methanol since other Mut$^-$ mutants were capable of inducing peroxisomes that were morphologically indistinguishable from those observed in wild-type cells (not shown). We concluded that these 8 mutant groups were peroxisome deficient (Per$^-$).

The eight Per$^-$ strains were examined genetically to determine whether Mut, Out and Per phenotypes were linked. Each mutant was crossed with an *arg4 P. pastoris* strain and 10 to 20 of the resulting spore progeny were selected at random and analyzed for

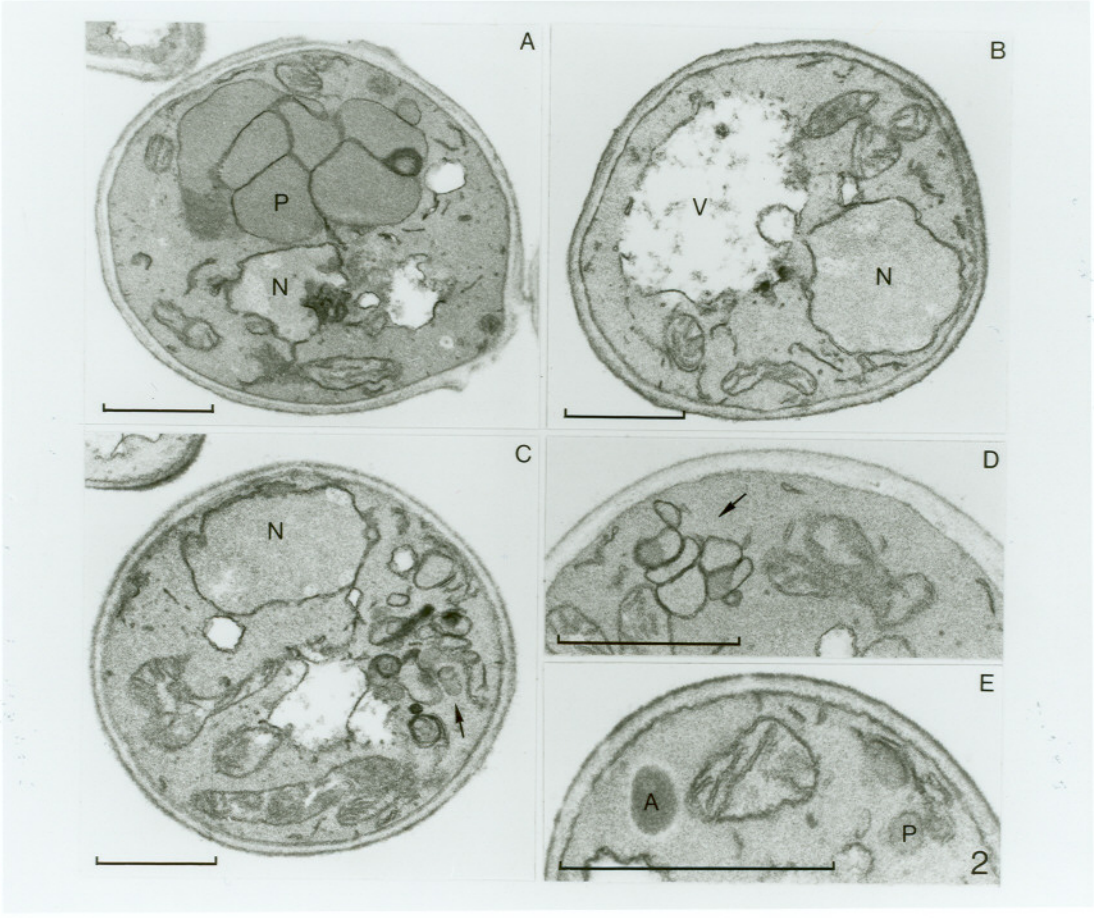

Figure 2.2 Morphology of selected methanol-induced *P. pastoris* strains. Each strain was precultured in YPD, transferred to methanol-containing medium and harvested after approximately 20 hours. Strains shown are: A, JC100 (wild type); B, JC101 (*per1*); C and D, JC111 (*per3*); E, JC112 (*per4*). Abbreviations are: N, nucleus; P, peroxisome; V, vacuole. Arrows point to abnormal membrane-bounded vesicles. Size bar represents 1 mM.

phenotype. All progeny from these crosses were either Mut$^-$ Out$^-$ Per$^-$ or Mut$^+$ Out$^+$ Per$^+$, a result which demonstrated that the three phenotypes were tightly linked and therefore, likely to be a consequence of a defect in a single gene in each mutant. These genes were named *PER1* through *PER8* and are listed along with their mutant alleles in Table 2.1.

2.4.3 Peroxisomal enzymes in *per* mutants

The presence of selected peroxisomal matrix enzymes was investigated. For these experiments, the 8 *per* mutants along with control strains were incubated in either methanol- or oleate-containing medium, prepared as cell-free extracts and assayed for catalase, alcohol oxidase (AOX) and acyl-CoA oxidase activities. Upon shifting to either medium, each *per* mutant induced approximately the same level of catalase as wild-type *P. pastoris* (Figures 2.3A and 2.3B). Acyl-CoA oxidase, a ß-oxidation pathway enzyme, reached specific activity levels in the *per* mutants that were only approximately 10% of those seen in wild-type *P. pastoris* (Figure 2.4A). However, their reduced activity could be attributed to a general inability to utilize oleate since Out$^-$ mutants with normal peroxisomes also displayed similarly reduced levels of acyl-CoA oxidase (Figure 2.4A).

AOX presented a more complicated picture. In wild-type *P. pastoris*, shift to methanol resulted in a rapid 1000-fold increase in AOX specific activity (Figure 2.4B). In contrast, AOX activity in each of the *per* mutants was much lower. At 4 hours after shift to methanol, the peak of activity in the *per* mutants, AOX activity ranged from 0.4% of wild-type to undetectable (< 0.0005 units). As a control for a general effect of carbon starvation on Mut$^-$ strains, activity was also measured in several Mut$^-$ but Per$^+$ *P. pastoris* mutants. AOX activity in these Per$^+$ strains was less than that in wild-type *P. pastoris* but not nearly as low as that observed in each of the *per* mutants (Figure 2.4B).

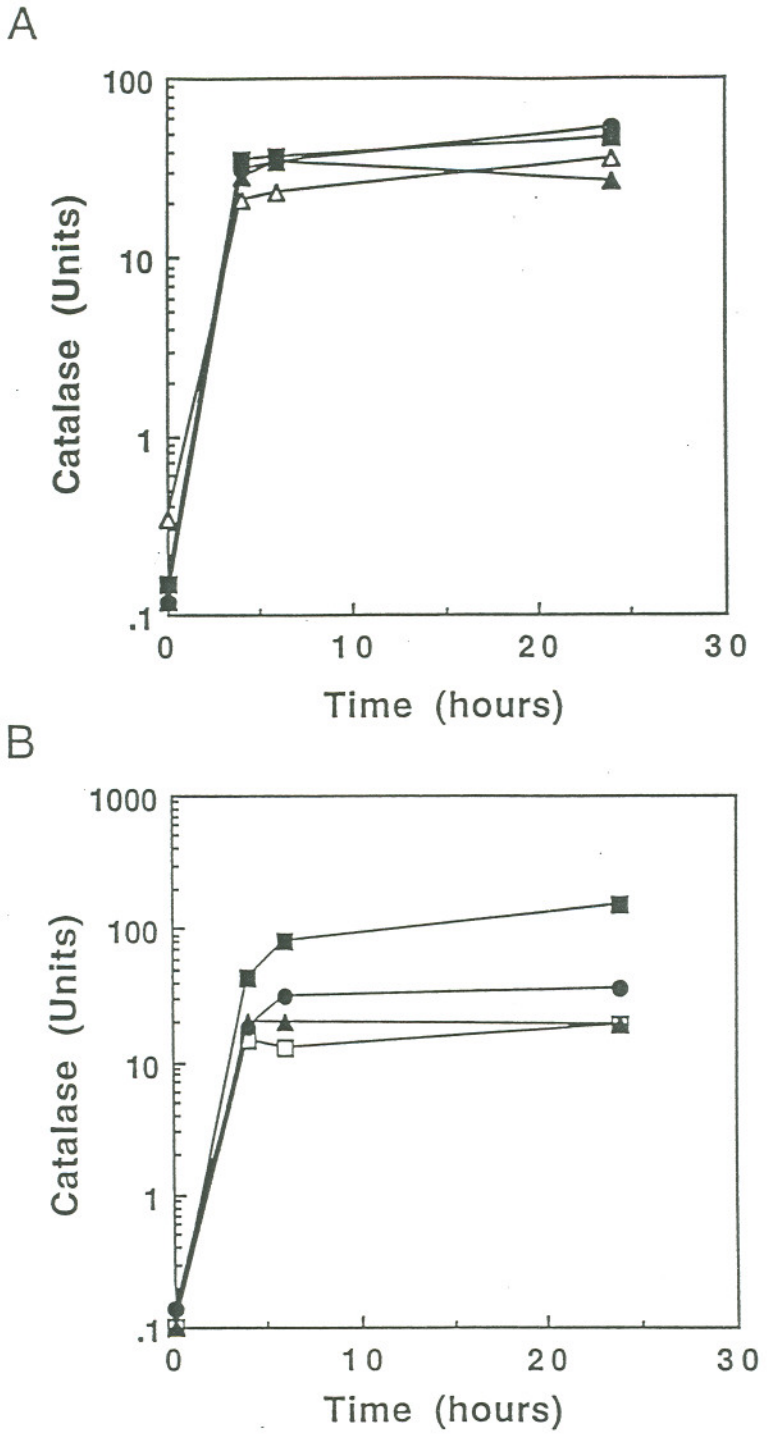

Figure 2.3. Induction of catalase activity in peroxisome-deficient *P. pastoris* strains. Graphs A and B show catalase activities in cell-free extracts of cultures incubated in methanol and oleate media, respectively. Time 0 is point at which cultures were shifted from YPD to induction media. Catalase activity is expressed as ΔE_{240} per min per mg protein. Strains shown are: JC100 (wild type),(■); MS105 (*fld*), (Δ); JC126 (*out1*), (□); JC101 (*per1*), (●); and JC105 (*per2*), (▲).

Active AOX is a homo-octomer which, in wild-type methylotrophic yeast cells, is transported into peroxisomes as an inactive monomer where it is then assembled into the active form (Goodman et al., 1984). It was possible that in our *per* mutants, AOX was synthesized but remained in an inactive form in the cytosol. To investigate whether inactive AOX might be accumulating in the mutants, cell-free extracts were prepared from methanol-induced cells and probed for AOX protein using anti-AOX polyclonal antibodies. Figure 2.5 shows an example of the results observed in these experiments. Each lane contained a 10-μg aliquot of extract (Figure 2.5, lower panel). Extracts prepared from *P. pastoris* wild-type and *fld* mutant strains (lanes 1 and 3; AOX specific activities of 1.1 and 0.15 units/mg protein, respectively) showed strong anti-AOX reacting bands at 72 kDa, the known molecular mass of AOX monomer (Ellis et al., 1985). (The relative band intensities in the two samples do not appear to be proportional to activity due to saturation of the filter by the wild-type sample.) As a negative control, an extract prepared from glucose-grown wild-type cells which contained no AOX activity showed no detectable AOX protein (lane 2). Finally, lanes 4 and 5 contained samples of extracts prepared from methanol-incubated cultures of *per1* and *per3* strains (AOX specific activities of 0.003 and 0.03 units/mg protein, respectively) and showed only very small amounts of AOX protein. Together, the immunoblot experiments revealed no significant differences between AOX activity and protein levels, suggesting that large amounts of inactive AOX protein were not present in the *per* mutants.

2.4.4 Subcellular localization of peroxisomal enzymes

Each *per* mutant was examined for the subcellular location of selected peroxisomal matrix enzymes by differential centrifugation. Methanol- or oleate-induced cells of each mutant and control strains were protoplasted, homogenized and, after a low-speed

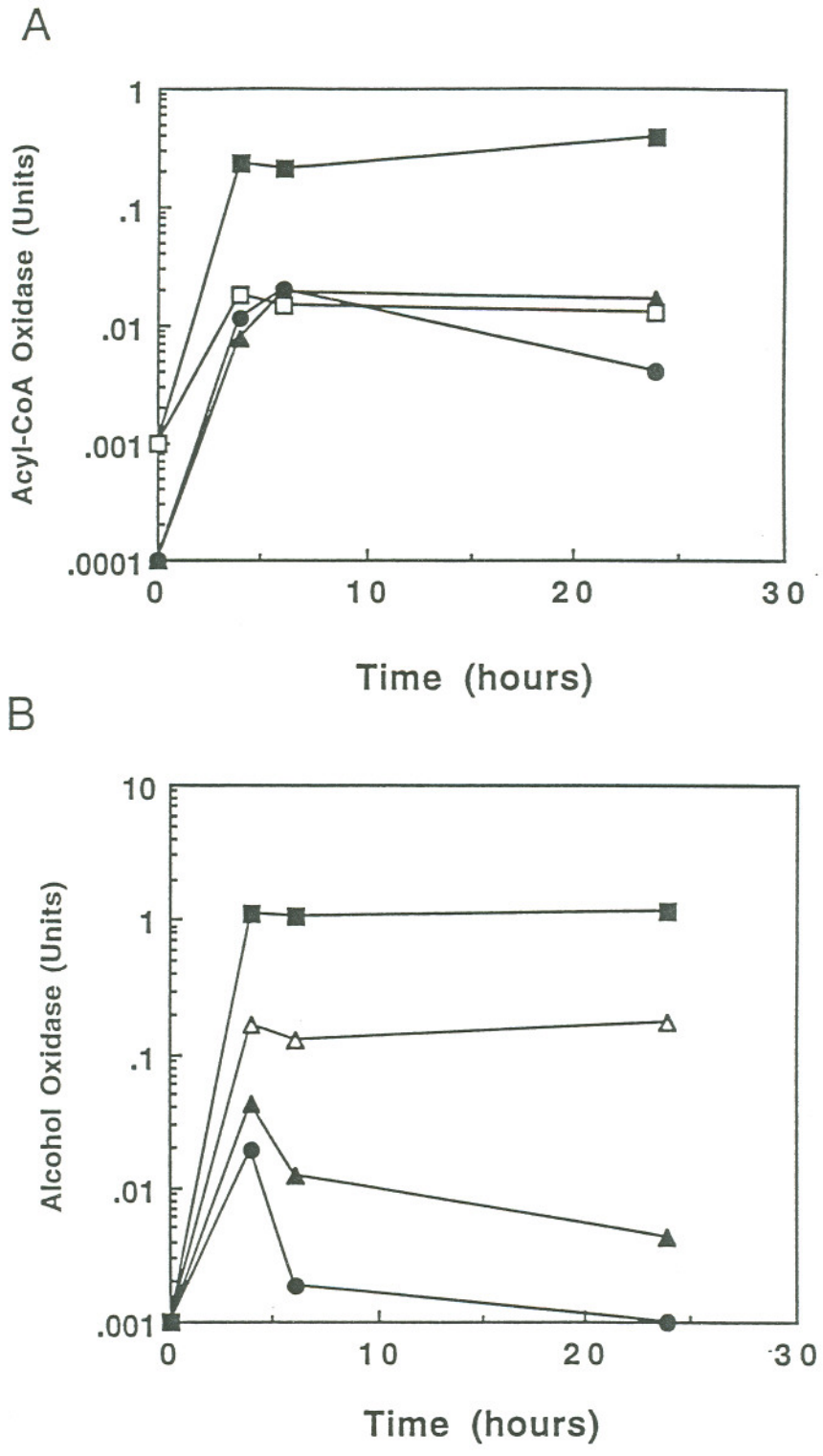

Figure 2.4. Induction of acyl-CoA oxidase and alcohol oxidase activities in *P. pastoris per* mutants. Graph A shows acyl-CoA oxidase activity at selected times after shift to oleate medium. Graph B presents alcohol oxidase activity after shift to methanol medium. Culture conditions and strain symbols are the same as described in the legend in Figure 2.3.

centrifugation to remove unbroken cells and other debris, were centrifuged at 20,000 x g, a force sufficient to sediment small organelles such as mitochondria and peroxisomes (Kamiryo et al., 1982). Enzyme-specific activities were then determined in supernatant (S) and pellet (P) fractions and expressed as a ratio (S:P). As shown in Table 2.2, catalase ratios from methanol- or oleate-induced cultures of Per^+ control strains were low, demonstrating that the majority of the enzyme was sedimentable. Addition of Triton X-100 to homogenates prior to centrifugation caused a dramatic shift of catalase to supernatant fractions, a result that suggested that sedimentable catalase was present within a membrane-enclosed vesicle. In contrast to control strains, *per* mutant ratios were high, suggesting that the enzyme was localized primarily in the cytosol. The high ratios did not appear to be a result of an increased sensitivity of *per* cells to the fractionation procedure or to unequal handling during preparation since activity ratios for fumarase, a mitochondrial marker enzyme, remained low.

Attempts to determine AOX activity ratios from mutant subcellular fractions were largely unsuccessful due to their low levels of activity (Table 2.2). However, it was clear that any AOX activity in *per* mutants was highly sedimentable. As observed for catalase, addition of Triton X-100 prior to 20,000 x g centrifugation resulted in release of AOX to supernatant fractions (Table 2.2). Thus, the small amount of AOX activity in *per* mutants appeared to be within a membrane-bounded structure.

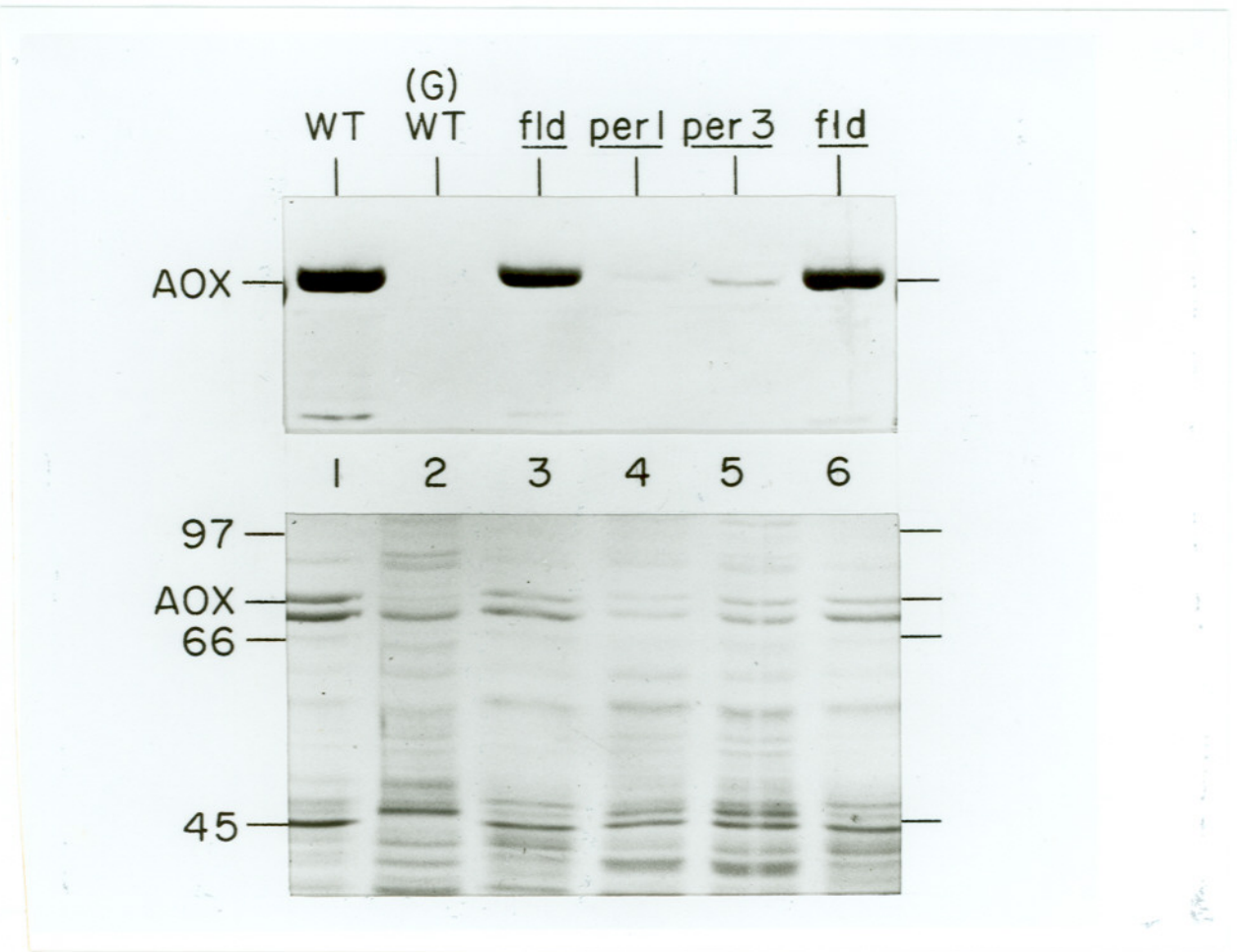

Figure 2.5. Detection of alcohol oxidase protein in cell-free extracts of methanol-induced *P. pastoris per* strains. Extracts were prepared, reduced, denatured and separated by electrophoresis through a 10% polyacrylamide gel. Each lane in the figure contains 10 µg of the following samples: lanes 1 and 2, methanol and glucose-grown wild-type *P. pastoris*, respectively; lanes 3 and 6, methanol-induced MS105 (*fld*); lanes 4 and 5, methanol-induced JC101 (*per1*) and JC111 (*per3*), respectively. The lower panel is a gel in which proteins were stained with Coomassie blue dye. The upper panel is an immunoblot of the same samples reacted with polyclonal anti-alcohol oxidase antibodies.

Table 2.2 Enzyme distribution after differential centrifugation[a]

Strain and treatment	Specific act[b] under following conditions[c]: Methanol Catalase	Methanol AOX	Methanol Fumarase	Oleate Catalase	Oleate Fumarase
JC100 (wild type)					
None	0.4	0.26	0.13	0.80	0.16
+ TX	5.2	4.8	5.2	30.5	8.2
MS105 (*fld*)					
None	0.27	0.16	1.0	-	-
+ TX	17.1	26.7	9.6	-	-
JC126 (*out1*)					
None	-	-	-	1.6	0.2
+ TX	-	-	-	25.3	17.3
JC101 (*per1*)					
None	9.5*	0.07*	0.10*	31.7	0.17
+ TX	12.0*	6.4*	2.8*	20.2	6.2
JC105 (*per2*)					
None	7.0*	0.04*	0.37*	16.3	0.28
+ TX	7.1*	15.1*	7.1*	17.9	10.6
JC111 (*per3*)	11.3	-	0.41	23.3	0.15
JC112 (*per4*)	6.2	-	0.42	22.0	0.03

JC113 (*per5*)	12.8	-	0.77	27.6	0.08
JC114 (*per6*)	8.1	-	0.54	24.2	0.34
JC115 (*per7*)	6.7	-	0.40	16.5	0.04
JC118 (*per8*)	10.1	-	0.33	27.8	0.17

[a] + TX, with Triton X-100 added prior to final centrifugation.

[b]Data are expressed as the ratio of the specific activities present in the 20,000 x g supernatant and pellet fractions; -, not determined.

[c]Cultures were induced in oleate medium for 5 hours or in methanol medium for 20 hours with the exception of those designated with an asterisk which were incubated for 5 hours in methanol medium.

2.5 Discussion

Our laboratory is interested in understanding peroxisome biogenesis and function at the molecular level. Toward that end, we have initiated a genetic approach using the methylotrophic yeast *Pichia pastoris* as a model system. In this report, we describe a critical tool required for these investigations, a rapid and efficient procedure for isolating peroxisome-deficient mutants (*per* mutants) of this yeast. In addition, we report the identification and initial characterization of a significant collection of *per* mutants. The key to the success of the mutant isolation scheme is the ability of *P. pastoris* to utilize two unusual carbon sources, methanol and oleic acid, both of which are dependent on peroxisomes for their metabolism (Erdmann et al., 1989; Cregg et al., 1990). By selecting for mutants that are simultaneously methanol- and oleate-utilization defective (Mut^- and Out^-, respectively), most mutants affected in non-peroxisomal genes are eliminated from consideration. The effectiveness of this screen is a major advantage since the primary method of identifying peroxisome-deficient mutants is direct examination of the organelles by electron microscopy, a process that is tedious and time-consuming when applied to large mutant collections. To our knowledge, *P. pastoris* is unique in having two easily observable growth phenotypes linked to the state of peroxisomes.

Another aspect of the *per* mutant isolation scheme is testing mutants for growth on other carbon sources such as ethanol or acetate. Peroxisome-deficient mutants have now been described in three yeast species--*S. cerevisiae*, *H. polymorpha* and *P. pastoris*--and all are competent in metabolism of C_2 substrates (Erdmann et al., 1989; Cregg et al., 1990; Sulter et al., 1991). This is somewhat surprising since C_2 growth requires glyoxylate-pathway enzymes which are located in peroxisomes (glyoxysomes). In *H.*

polymorpha, glyoxysomes are clearly affected in *per* mutants since pathway enzymes, isocitrate lyase and malate synthase, are mislocalized to the cytosol (Cregg et al., 1990; Sulter et al., 1991). The continued ability of *per* mutants to utilize ethanol and other carbon sources is particularly useful in eliminating regulatory mutants that are defective in ability to derepress alternate carbon source utilization pathways. Such mutants fail to proliferate peroxisomes when induced and therefore, appear to be peroxisome deficient (Simon et al., 1992).

We applied this phenotypic screen to a collection of 280 Mut$^-$ *P. pastoris* mutants representing approximately 46 complementation groups and found 10 groups that are Mut$^-$ and Out$^-$ but capable of growth on ethanol and glucose. Electron microscopic examination of methanol-induced cells of representative strains from these groups revealed that 8 are grossly deficient in peroxisomes (Per$^-$) while 2 contain normal-appearing peroxisomes (Per$^+$). The existence of Mut$^-$, Out$^-$ but Per$^+$ strains suggests that genes may exist whose products are specifically required for growth on methanol and oleate but are not directly involved in peroxisome function. On the other hand, it may be that these mutants are affected in peroxisome function but in a way that does not alter the morphology of the organelle. More importantly, considering that 8 of 10 groups are *per* mutants, the screen is remarkably efficient. Additional *P. pastoris* peroxisome-deficient mutants have been isolated using this screen (Subramani and Gould, 1992) and complementation results indicate that the 2 collections represent mutations in 12 different peroxisomal genes (J. Cregg, unpublished data).

Subcellular fractionation experiments provided independent evidence that the 8 *per* mutants are peroxisome deficient. In both methanol- and oleate-induced cells of each mutant, catalase activity was located almost entirely in the 20,000 x g supernatant fraction, a result that suggests the enzyme is mislocalized to the cytosol. In contrast,

catalase activity is present primarily in 20,000 x g pellet fraction in samples prepared from Per$^+$ control strains. It should be noted that, even in these control strains, a significant portion of catalase was consistently present in supernatant fractions. This may be an artifact caused by peroxisome leakage or breakage during the fractionation procedure. In *H. polymorpha*, similar catalase ratios are obtained despite the fact that catalase is exclusively peroxisomal in this yeast (van Dijken et al., 1975). However, it is also possible that, like *S. cerevisiae*, *P. pastoris* harbors both cytosolic and peroxisomal catalases (van der Klei et al., 1990).

Interestingly, alcohol oxidase (AOX), a peroxisomal methanol-pathway enzyme, is virtually absent in methanol-induced cells of each *per* mutant. Precedents for this result have been reported for certain matrix enzymes in peroxisome-deficient Chinese hamster ovary (CHO) cells and Zellweger fibroblasts (Suzuki et al., 1986; Zoeller and Raetz, 1986). For two of these enzymes, acyl-CoA oxidase and thiolase, pulse-labeling experiments indicated that the enzymes are synthesized but, as a consequence of their failure to be imported, are rapidly degraded in the cytosol (Schram et al., 1986; Suzuki et al., 1986). If this is also the case with AOX in our *per* mutants, it would be the first description of this phenomenon in a yeast. Experiments aimed at examining the fate of AOX in the *per* mutants are in progress.

The small amount of AOX that is present in *per* mutants is sedimentable. Furthermore, treatment of *per* cell homogenates with Triton X-100 prior to centrifugation releases AOX into the supernatant, a result that suggests the enzyme is present within a membrane-bound structure. The simplest explanation for these observations is that the *per* mutations are slightly "leaky" and as a result, a small amount of AOX is imported into a few remaining peroxisomes where it assembles into active enzyme.

The *P. pastoris per* mutants share a number of similarities with peroxisome-deficient

mutants described in *S. cerevisiae*, *H. polymorpha*, Chinese hamster ovary (CHO) and human Zellweger cell lines (Zoeller and Raetz, 1986; Erdmann et al.; Cregg et al., 1990). First, in all five species, peroxisome deficiency results from recessive mutations in any one of a number of different genes. Second, catalase as well as certain other matrix enzymes are active but appear to be mislocalized to the cytosol. Third, normal peroxisomes are virtually absent from the cells and in their place, abnormal single-membrane-enclosed vesicles are observed. In peroxisome-deficient CHO mutants and in Zellweger cell lines, it has been shown that these vesicles are without most matrix enzymes but do contain peroxisomal membrane proteins (Lazarow et al., 1986; Zoeller and Raetz, 1986; Santos et al., 1988a; Santos et al., 1988b; Tsukamoto et al., 1990). The presence of these peroxisomal remnants, termed "ghosts," has lead to the idea that the mutants are defective in a major matrix protein import system (Santos et al., 1988b). We have yet to establish whether the vesicles we observe in the *P. pastoris per* mutants are the equivalent of peroxisomal "ghosts."

In summary, the mechanisms responsible for peroxisome biogenesis remain a major unsolved mystery in cell biology. We believe that a combined genetic and biochemical attack is best able to solve that mystery. We have demonstrated that mutants with defects in peroxisome function are easily isolated in the yeast *P. pastoris* due to the fortuitous presence of two peroxisome-dependent carbon source utilization pathways in this organism. The existence of *P. pastoris per* mutants along with efficient transformation vectors and host strains for this yeast have enabled us to take the next major step in our studies, the cloning of *P. pastoris PER* genes by complementation. To date, we have isolated genes *PER3*, *PER4* and *PER6* (J. Cregg, unpublished data). The DNA sequences of these and other *PER* genes are expected to provide useful insights into the function of their products and their roles in peroxisome biogenesis.

CHAPTER 3

PER3, A GENE REQUIRED FOR PEROXISOME BIOGENESIS IN *PICHIA PASTORIS*, ENCODES A PEROXISOMAL MEMBRANE PROTEIN INVOLVED IN PROTEIN IMPORT

3.1 Summary

PER genes are essential or the biogenesis of peroxisomes in the yeast *Pichia pastoris*. Here we describe the cloning of *PER3* and functional characterization of its product Per3p. The *PER3* sequence predicts that Per3p is a 713 amino acid (81 kDa) hydrophobic protein with two membrane-spanning domains. We show that Per3p is a membrane protein of the peroxisome. Methanol- or oleate-induced cells of *per3-1*, a mutant strain generated by chemical mutagenesis, lack normal peroxisomes but contain numerous abnormal vesicular structures. The vesicles contain thiolase, a PTS2 protein, but only a small portion of several other peroxisomal enzymes, including heterologously expressed luciferase, a PTS1 protein. These results suggest that the vesicles in *per3-1* cells are peroxisomal remnants similar to those observed in cells of patients with the peroxisomal disorder Zellweger syndrome and that the mutant is deficient in PTS1 but not PTS2 import. In a strain in which most of *PER3* was deleted, peroxisomes as well as peroxisomal remnants appeared to be completely absent, and both PTS1- and PTS2-containing enzymes were located in the cytosol. We propose that Per3p is an essential component of the machinery required for import of all peroxisomal matrix proteins and is composed of independent domains involved in the import of specific PTS groups.

3.2 Introduction

Peroxisomes (glyoxysomes, glycosomes) are ubiquitous, eukaryotic organelles that are enclosed by a single membrane and are the site of hydrogen peroxide-generating oxidases as well as catalase which decomposes this toxic byproduct into water and oxygen. An unusual feature of peroxisomes is that the specific pathways that involve the organelles vary greatly depending upon the organism (Fahimi and Sies, 1987; van den Bosch et al., 1992). Furthermore, their size, number, and enzymatic content within a single organism or tissue can change dramatically in response to environmental stimuli (Veenhuis and Harder, 1991; Green, 1992). Peroxisomes have no DNA and, therefore, all peroxisomal proteins are likely to be encoded by nuclear DNA (Kamiryo et al., 1982). Proteins destined for the peroxisome are synthesized on free polysomes and post-translationally imported into the organelle in an ATP-dependent manner (Fujiki et al., 1984; Imanaka et al., 1987; Wenderland and Subramani, 1993). Two peroxisomal targeting signals (PTS) have been characterized in detail (Subramani, 1993). The first, PTS1, is a tripeptide motif, Ser-Lys-Leu (and conservative variants), that is located at the carboxyl terminus of many peroxisomal matrix proteins (Gould et al., 1987; Swinkels et al., 1992). Proteins that end in PTS1 are properly imported into peroxisomes of animals and fungi, glyoxysomes of plants, and glycosomes of trypanosomes (Gould et al., 1990; Keller et al., 1991). Thus, the PTS1 system has been conserved throughout evolution. The second, PTS2, is characterized by the consensus sequence (RL/IX$_5$Q/HL) that is located near the amino-terminus of some peroxisomal proteins including: 3-ketoacyl-CoA thiolases from rats, humans, and several yeast species; watermelon malate dehydrogenase; and amine oxidase and the peroxisome biogenesis protein Perp1 from the yeast *Hansenula polymorpha* (Osumi et al., 1991; Swinkels et al., 1991; de Hoop and AB, 1992; Gietl et al., 1994; Glover et al., 1994b; Waterham et al., 1994; Faber et al.,

1995). Evidence suggests that additional PTSs exist internally within some peroxisomal proteins including: *Candida tropicalis* acyl-CoA oxidase (Kamiryo et al., 1988; Small et al., 1989) , *Saccharomyces cerevisiae* catalase (Kragler et al., 1993), and *Candida boidinii* 47-kDa peroxisomal membrane protein PMP47 (McCammon et al., 1994), and *H. polymorpha* malate synthase (Bruinenberg et al., 1990).

In humans, peroxisomes are indispensable for survival as demonstrated by a family of lethal genetic disorders collectively referred to as Zellweger syndrome in which normal peroxisomes appear to be absent (Lazarow and Moser, 1989). In cell lines derived from these patients, peroxisomal enzymes remain in the cytosol and are either present and active or absent due to rapid degradation (Wanders et al., 1984; Wiemer et al., 1989). Cell fusion studies with Zellweger cell lines indicate that the disease is the consequence of defects in any one of at least nine different genes (Shimozawa et al., 1992; Yajima et al., 1992). Recently human cDNA clones were identified that restored normal organelles to two different Zellweger complementation groups (Shimozawa et al., 1992; Gärtner et al., 1992). Although normal peroxisomes are absent from or grossly deficient in Zellweger cells, abnormal vesicles are observed (Santos et al., 1988a; Santos et al., 1988b). These vesicles contain peroxisomal membrane proteins and, thus, are believed to be peroxisomal remnants, termed ghosts. Furthermore, the ghost structures in some Zellweger lines have been shown to contain the PTS2 enzyme thiolase, suggesting that these lines have a functional PTS2 import system and must be defective in one or more other import systems (Balfe and Hoefler, 1990; Motley et al., 1994).

We have selected the methylotrophic yeasts *Pichia pastoris* and *Hansenula polymorpha* as a model systems to study the molecular mechanisms involved in the biogenesis of peroxisomes. In *P. pastoris*, peroxisome-deficient (*per*) mutants have been isolated that are affected in 10 different genes (*PER* genes) (Liu et al., 1992; Gould et

al., 1992). Each *per* mutant is defective in ability to grow on either methanol or oleic acid, whose metabolism requires multiple peroxisomal enzymes, but is capable of normal growth on other substrates such as glucose, ethanol, and glycerol. Like Zellweger cells, *P. pastoris per* mutants lack normal peroxisomes and contain cytosolic peroxisomal matrix enzymes, e.g., catalase. In addition, alcohol oxidase, an abundant peroxisomal enzyme required for methanol metabolism, is present at greatly reduced levels in methanol-induced *per* mutant cells, a phenomenon that is observed with certain peroxisomal enzymes in Zellweger cell lines. Finally, similar to Zellweger cells, methanol- or oleate-induced *per* cells contain abnormal single and multiple membrane-bound vesicles. We have utilized the *per* mutants to clone *PER* genes by functional complementation. We report that *PER3* encodes a peroxisomal integral membrane protein that appears to be involved in peroxisomal protein import. Furthermore, we show that the abnormal vesicles observed in a *per3* mutant contain peroxisomal matrix enzymes such as thiolase, suggesting that these structures are peroxisomal in origin.

3.3 Materials and Methods

3.3.1 Strains, Media and Microbial Techniques

P. pastoris strains used in this study are listed in Table 3.1. *P. pastoris* cultures were grown at 30°C in a complex medium (YPD) composed of 1% yeast extract, 2% peptone, and 2% dextrose, or in one of the following defined media: YNB medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate) supplemented with either 0.4% dextrose, 0.5% methanol, or 0.2% oleate, 0.02% Tween 40, 0.05% yeast extract. Nutritional supplements for growth of auxotrophic strains were added to 50 mg/ml as required. Sporulation (mating) medium as well as procedures for genetic manipulations of *P. pastoris* have been described (Liu et al., 1992). *P. pastoris* transformations were performed as described in Cregg et al. (1985). Vector pJAH23, a gift from Dr. S. Subramani (University of California at San Diego) is composed of the firefly luciferase gene under the transcriptional control of the *P. pastoris AOX1* promoter and terminator, the *S. cerevisiae ARG4* gene, the *P. pastoris* autonomous replication sequence PARS2 and bacterial plasmid pBR322.

3.3.2 Subcellular Fractionation

P. pastoris strains were precultured in 1 liter of YPD medium to an OD_{600} of approximately 0.4 and shifted by centrifugation to 1 liter of YNB medium containing either methanol or oleic acid as sole carbon source. After 6 h, cultures were harvested by centrifugation at 5,000 x g for 10 min at 4°C. Cells were washed 4 times with 100 ml of distilled water and suspended in 10 ml of spheroplasting buffer [5 mM K 3-(N-morpholino) propanesulfonate, pH 7.2, 0.5 M KCl, 10 mM Na_2SO_3]. Cells were converted to spheroplasts by the addition of 0.8 mg of Zymolyase 100T (ICN, Costa Mesa, CA) and incubation at 30°C for 30 min. Spheroplasts were centrifuged at 2,500 x

Table 3.1 Yeast strains

Strain	Genotype	Source or reference
JC100	Wild type	NRRL Y-11430
GS115	*his4*	Cregg et al. (1985)
GS190	*arg4*	Cregg et al. (1989)
JC111	*per3-1*	Liu et al. (1992)
JC120	*per3-1 his4*	This study
JC121	*per3Δ::SARG4 arg4*	This study
JC122	*per3-1 arg4*	This study
JC123	*arg4* (pJAH23)	This study
JC124	*per3-1 arg4* (pJAH23)	This study
JC125	*per3Δ:::SARG4 arg4* (pJAH23)	This study

g for 8 min and gently resuspended in 5 ml of sorbitol-MES buffer [5 mM K-2-(N-morpholino) ethanesulfonic acid, pH 6.0, 0.5 mM EDTA, 0.6 M sorbitol, 0.1% ethanol] supplemented with proteinase inhibitors at final concentrations of 1 mM PMSF, 5 mg/ml aprotinin, 2.5 mg /ml leupeptin, and 0.21 mg/ml NaF. The mixture was homogenized by 10 strokes with a Potter-Elvehjem tissue homogenizer and centrifuged at 2,000 x g for 10 min and 4°C to remove cell debris and unlysed cells, and the supernatant was collected and centrifuged at 20,000 x g for 20 min and 4°C to sediment small organelles. For differential centrifugation analysis of the resulting crude organelle pellet and supernatant fractions, the final pellet was resuspended in 0.5 ml of sorbitol-MES buffer supplemented with the proteinase inhibitors. For sucrose density gradients, the final pellet was resuspended in 5.0 ml of the same buffer.

Peroxisomes were purified by separation through discontinuous sucrose gradient as described (Kamiryo et al., 1982). Enriched organelle preparations were layered on top of gradients composed of the following volumes and concentrations (wt/wt) of sucrose in 5 mM MES, pH 6.0, 0.5 mM EDTA buffer: 6 ml of 53%, 12 ml of 42%, 6 ml of 35%, and 4 ml of 25%. Gradients were centrifuged at 4°C for 6 h at 27,000 rpm in a Beckman SW27 rotor. Gradient fractions of 1.3 ml were collected manually and assayed immediately for enzymatic activities. Equal volumes of each fraction were prepared for SDS-PAGE and immunoblotting by precipitation in 10% trichloroacetic acid (TCA) for 2 h on ice and centrifugation at 4°C for 10 min at 16,000 x g in an Eppendorf minicentrifuge. The resulting protein pellets were washed 2 times by suspension in acetone and centrifugation as described above. The final pellets were suspended in 30 ml of Laemmli sample buffer with 5% ß-mercaptoethanol. Samples were boiled for 5 min and stored at -20°C.

3.3.3 *P. pastoris* DNA Library Construction

The *P. pastoris* genomic DNA library used to clone *PER3* was constructed in *P. pastoris-Escherichia coli* shuttle vector pYM8 (Cregg et al., 1985). This vector is composed of a *P. pastoris* autonomous replication sequence (PARS1), the *Saccharomyces cerevisiae* histidinol dehydrogenase gene (*HIS4*), which is a selectable marker for transformation of *his4* strains of *P. pastoris*, and sequences from *E. coli* plasmid pBR322. To construct the library, pYM8 was digested with BamHI and treated with calf intestine alkaline phosphatase (CIP). Total DNA was extracted from wild-type *P. pastoris*, and partially digested with Sau3AI to generate DNA fragments of 5-10 kilobase pairs (kb). Fragments in this size range were further size selected by sucrose gradient centrifugation. Approximately equal molar amounts of *Sau*3AI-digested yeast DNA and *Bam*HI-CIP-treated pYM8 were ligated using T4 DNA ligase and transformed into *E. coli* strain MC1061 by selection for ampicillin resistance. Plasmid DNA was extracted from approximately 56,000 transformed colonies by the alkaline lysis procedure and further purified by centrifugation in an ethidium bromide/cesium chloride density gradient (Sambrook et al., 1989). The library was stored as plasmid DNA in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA buffer at -20°C.

3.3.4 Isolation and Characterization of *PER3*

P. pastoris strain JC120 (*per3-1 his4*) was transformed with 5 µg of the above *P. pastoris* library by the spheroplast-generation $CaCl_2$-polyethylene glycol fusion method, and transformants were selected for His^+ prototrophy on YNB glucose medium (Cregg et al., 1985). The approximately 50,000 transformants that resulted were collected from the plates as a single pool and inoculated into liquid YNB methanol medium at an initial OD_{600} of 0.1. After 3 days, the culture had grown on methanol and was harvested. Total

DNA was extracted from these cells and transformed into *E. coli* strain MC1061. Plasmid DNA was extracted from several resulting transformants and examined by cleavage with selected restriction enzymes. All recovered vectors appeared to be identical with inserts of approximately 8.1 kb. One of the vectors, pYT4, was retransformed into JC120, was observed to cotransform cells to both Mut^+ and His^+ with approximately equal efficiency and was selected for further study (Fig.3.4). To define the location of *PER3*, selected subfragments from pYT4 were subcloned and tested for their ability to complement JC120 for growth on methanol. In addition, selected segments of pYT4 were removed, and the truncated vectors religated and tested for complementation. Results indicated that the complementing activity was located within a 5-kb region between *Sca*I and the *Bam*HI/*Sau*3AI junction. Northern blot studies using subfragments within this region indicated that a 3.3-kb *Eco*RV-*Sca*I fragment transcribed a methanol-induced 2.1-kb mRNA. Two subfragments from this region, a 2.6-kb *Sca*I-*Hind*III fragment and a 0.7-kb *Xba*I-*EcoR*V fragment were inserted into appropriately digested pBluescript II SK+ (Stratagene, La Jolla, CA), and a series of nested deletions was generated from each of the resulting plasmids by limited digestion with exonuclease III as described previously (Sambrook et al., 1989). The DNA sequence of both DNA strands was then determined by the dideoxy method (Sanger et al., 1977) using Sequenase 2.0 (USB, Cleveland, OH). DNA and predicted protein sequences were analyzed using MacVector software (IBI, New Haven, CT). Sequencing results revealed a long open reading frame whose 5' terminus extended beyond the *EcoR*V site. To to complete the DNA sequence, a series of extending oligonucleotide primers were synthesized and utilized in sequencing reactions along with pYT4 DNA as template. The predicted amino acid sequence of the open reading frame was compared with those in the GenBank data base.

3.3.5 Construction of the *PER3* Disruption Strain

A vector designed to delete most of *PER3* was constructed in two steps starting with plasmid pYM25. pYM25 is composed of a 3.1-kb *Hind*III fragment encoding the *S. cerevisiae ARG4* gene inserted into the *Hind*III site of pBR322. In the first step, a 2.0-kb EcoRI fragment composed of sequences flanking the 5' terminus of *PER3* was inserted into the *Eco*RI site of pYM25 to create plasmid pYH2. Second, a 1.5-kb PvuII fragment composed of 3' *PER3* sequences was inserted into the *Nru*I site of pYH2 to create the *PER3*-deletion vector pYH3. The *PER3* locus in the vector contains a deletion of 1638 bp of *PER3* coding sequence (amino acids 1-546) with the *S. cerevisiae ARG4* gene fragment inserted at the deletion site (Fig. 3.4). pYH3 was linearized by partial digestion with *Pvu*II and transformed into GS190 (*arg4*). Arg^+ transformants were selected and screened for ones that were Mut^-. Several independent Arg^+ Mut^- transformants were collected and examined by the Southern blot method to confirm proper insertion of the linear fragment from pYH3 into the *PER3* locus.

3.3.6 Preparation of Anti-Per3p Antibodies

Per3p 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 *PER3*-containing DNA fragment in the proper reading frame by digesting the vector with *EcoR*I and inserting an adaptor oligonucleotide (AATTGAATGCATTTC). The adaptor insertion resulted in the introduction of an *Nsi*I site that was in frame with an *Nsi*I site in *PER3*. A 1.0-kb *Nsi*I-*Bgl*II fragment from pYH1 encoding amino acids 479-713 of *PER3* was then inserted into *Nsi*I- and *Bam*HI-digested pMAL-NsiI vector to create the *malE-PER3* expression vector, pEH1. MAL-Per3p fusion protein synthesis in *E. coli* strain TB1 transformed with pEH1

and purification were performed as recommended by the supplier.

Polyclonal antibodies against the fusion protein were raised in rabbits (Josman Laboratories, San Jose, CA). Anti-Per3p antiserum was affinity purified by the procedure described by Raymond et al. (1990). Crude rabbit serum was passed twice through a column containing total soluble *E. coli* protein from induced cells of strain TB1 (pMAL-c2). The flow-through from this column was then passed twice through a column to which total soluble protein from strain JC121 (*per3Δ:::SARG4 arg4*) had been bound. The flow-through from this column was collected and used at a 1:1,000 dilution.

3.3.7 Protein Extraction from Peroxisomes

Protein was extracted from peroxisomal membranes using either triethanolamine (TEA) or carbonate (Sulter et al., 1993; Fujiki et al., 1982). For TEA extractions, samples of approximately 25 µg of protein of purified peroxisomes from sucrose gradients were diluted 5 fold into 20 mM TEA, pH 7.8, containing the proteinase inhibitor mix and were incubated on ice for 1 h with occasional mixing. Samples were then centrifuged at 30,000 x g for 30 min at 4°C. Protein in the resulting supernatant was precipitated in 10% TCA, washed twice with acetone and suspended in SDS-PAGE buffer with 5% ß-mercaptoethanol. Pellet fractions were suspended in 30 ml of SDS-PAGE buffer with 5% ß-mercaptoethanol.

For sodium carbonate extractions, approximately 25 µg of protein from peroxisomal fractions was diluted 4 fold with MES buffer containing the proteinase inhibitor mix. A 0.5 M sodium carbonate solution, pH 11.5, was added to a final concentration of 100 mM, and the mixture was incubated on ice for 1 h with occasional mixing. Samples were centrifuged at 4°C for 2 h at 200,000 x g using a Beckman Type 65 rotor. Supernatant and pellet samples were then prepared for SDS-PAGE as described above.

3.3.8 Miscellaneous Methods

The *E. coli* strain used for recombinant DNA manipulations was MC1061 (Sambrook et al., 1989), except for expression of *malE-PER3* where strain TB1 was employed (New England Biolabs, Beverly, MA). *E. coli* cultivation and recombinant DNA techniques were performed as described (Sambrook et al., 1989). Total protein was determined by Bradford assay using bovine serum albumin as standard (Bradford 1976). Catalase (Ueda et al., 1990), acyl-CoA oxidase (Dommes et al., 1981), alcohol oxidase (van der Klei et al., 1990), luciferase (Nguyen et al., 1988), cytochrome c oxidase (Douma et al., 1985) and fumarase (Tolbert 1981) activities were measured according to published procedures. SDS-PAGE and immunoblotting procedures were performed as previously described (Laemmli, 1970; Sambrook et al. 1989). For immunoblotting experiments, rabbit polyclonal antibodies against *S. cerevisiae* thiolase (a gift of W.-H Kunau, Ruhr-Universitat, Bochum, Germany), *H. polymorpha* catalase, and *P. pastoris* alcohol oxidase (a gift of M. Gleeson, SIBIA, San Diego) were used. Antigen antibody complexes were visualized using the ECL kit following the instructions of the supplier (Amersham, Arlington Heights, IL). Electron microscopy and immunocytochemistry with rabbit antibodies raised against *Candida tropicalis* thiolase (a gift of R. Rachubinski, University of Alberta, Edmonton, Canada) and *Hansenula polymorpha* alcohol oxidase were performed as described (Waterham et al., 1992).

3.4 Results

3.4.1 *per3* Cells are Defective in Peroxisome Biogenesis

P. pastoris strain JC111 (*per3-1*) was isolated from a mutagenized culture by screening for strains that were defective in the utilization of both methanol (Mut^-) and oleic acid (Out^-) as sole carbon and energy sources (Liu et al., 1992). These substrates were selected because each requires several peroxisomal enzymes as well as functional peroxisomes to be metabolized (Veenhuis and Harder, 1991). In methanol- or oleate-grown wild-type cells, numerous large peroxisomes were readily observed (Fig. 3.1A, B). In contrast, normal peroxisomes were absent from methanol- or oleate-induced *per3-1* cells (Fig. 3.1C). Instead, clusters of membranous vesicles were frequently observed.

Evidence for a defect in peroxisome biogenesis in *per3-1* was obtained from biochemical and cytochemical experiments designed to determine the location of peroxisomal enzymes. Cell-free extracts of induced *per3-1* cells were examined for activities of selected peroxisomal enzymes. In *per3-1* cells induced with either methanol or oleate, catalase (CAT) activity was present at approximately the same level as wild-type cells (Fig. 3.2A). In contrast, activity for alcohol oxidase (AOX), the first enzyme in the methanol catabolism and a major constituent of the peroxisome matrix of methanol-grown cells, was consistently present at levels that were less than 3% of those in wild-type *P. pastoris*. Initially, AOX protein levels also appeared to be low, as judged by immunoblots using anti-AOX antibodies. It was possible that AOX was synthesized in *per3-1* cells but, due to inefficient import, remained mostly in the cytosol as insoluble aggregates which may have sedimented during preparation of cell free extracts. To examine this possibility, total cell extracts (prepared without centrifugation), were prepared and assayed for AOX activity and protein. Results revealed that methanol-

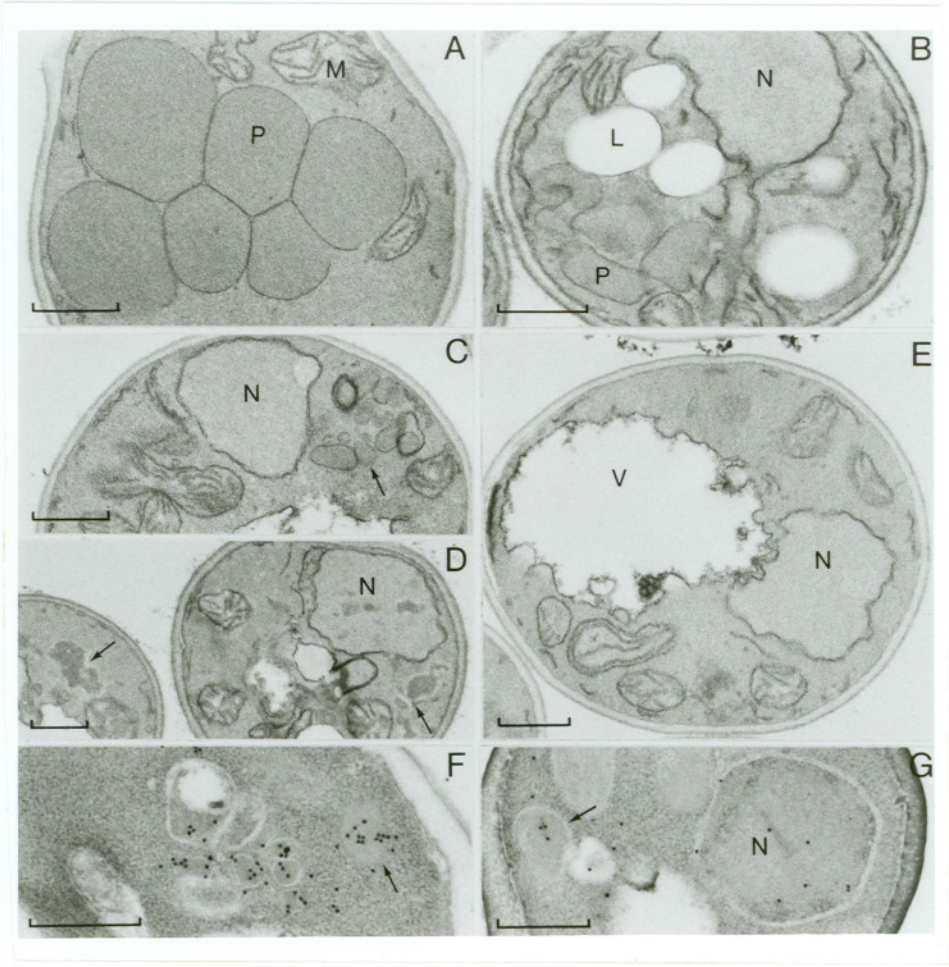

Figure 3.1. Electron micrographs showing subcellular morphology and location of selected peroxisomal enzymes in induced *per3* cells. Peroxisomes in wild-type cells grown on methanol (A) and oleate (B). In methanol-induced *per3-1* cells, peroxisomal remnants are evident (C, arrow); these cells also contain electron-dense aggregates (D, arrows). In methanol-induced cells of per3Δ strain, peroxisomal remnants are absent (E). Immunocytochemically, AOX protein is observed in peroxisomal remnants and cytosolic aggregates in methanol-induced *per3-1* (F, arrow). CAT is also found in these vesiceles (G, arrow) and in the cytosol and nucleus (G) (A-E, MnO_4-fixed cells; F-G, aldehyde/Unicryl/uranyl acetate. Abbreviations: L, lipid droplet; M, mitochondrion; N, nucleus; P, peroxisomes; V, vacuole. Bar, 0.5 μm.

induced *per3-1* cells actually contained approximately the same amount of AOX protein as wild-type cells (Fig. 3.2A). However, AOX activity levels remained low indicating that most of the AOX protein was inactive. Further evidence of AOX aggregate formation in methanol-induced *per3-1* was provided by electron microscopy (EM) studies, which showed that cytosolic aggregates were common in these cells (Fig. 3.1D) and immunocytochemical experiments which showed that AOX was concentrated in these aggregates (Fig. 3.1F).

To determine the location of selected peroxisomal enzymes in *per3-1* cells, differential centrifugation experiments were performed on homogenized spheroplast prepared from induced cells. With either methanol- or oleate-grown wild-type cells, a large portion of CAT activity was present in the 20,000 x g pellets (Fig. 3.2B). In contrast, in *per3-1* cells induced with either substrate, CAT was located primarily in the supernatant, indicating that the enzyme was cytosolic. As a control, cytochrome c oxidase, a mitochondrial marker enzyme, remained mostly in the pellet in *per3-1* preparations. Interestingly, the small amount of AOX activity remaining in methanol-induced *per3-1* cells was present in the pellet fractions (Fig. 3.2B). This suggested that our *per3-1* allele may not be totally defective in AOX import and, as a consequence, peroxisomes may be capable of importing a small fraction of the AOX protein that is synthesized where it assembles into active enzyme. Evidence that a portion of AOX actually was imported was obtained by immunocytochemistry which showed that, in addition to aggregates as described above, AOX was also present within the small vesicular structures in *per3-1* cells (Fig. 3.1F). Identical results were also obtained for CAT (Fig. 3.1G) and dihydroxyacetone synthase (DHAS), the third peroxisomal enzyme in the methanol catabolic pathway (data not shown).

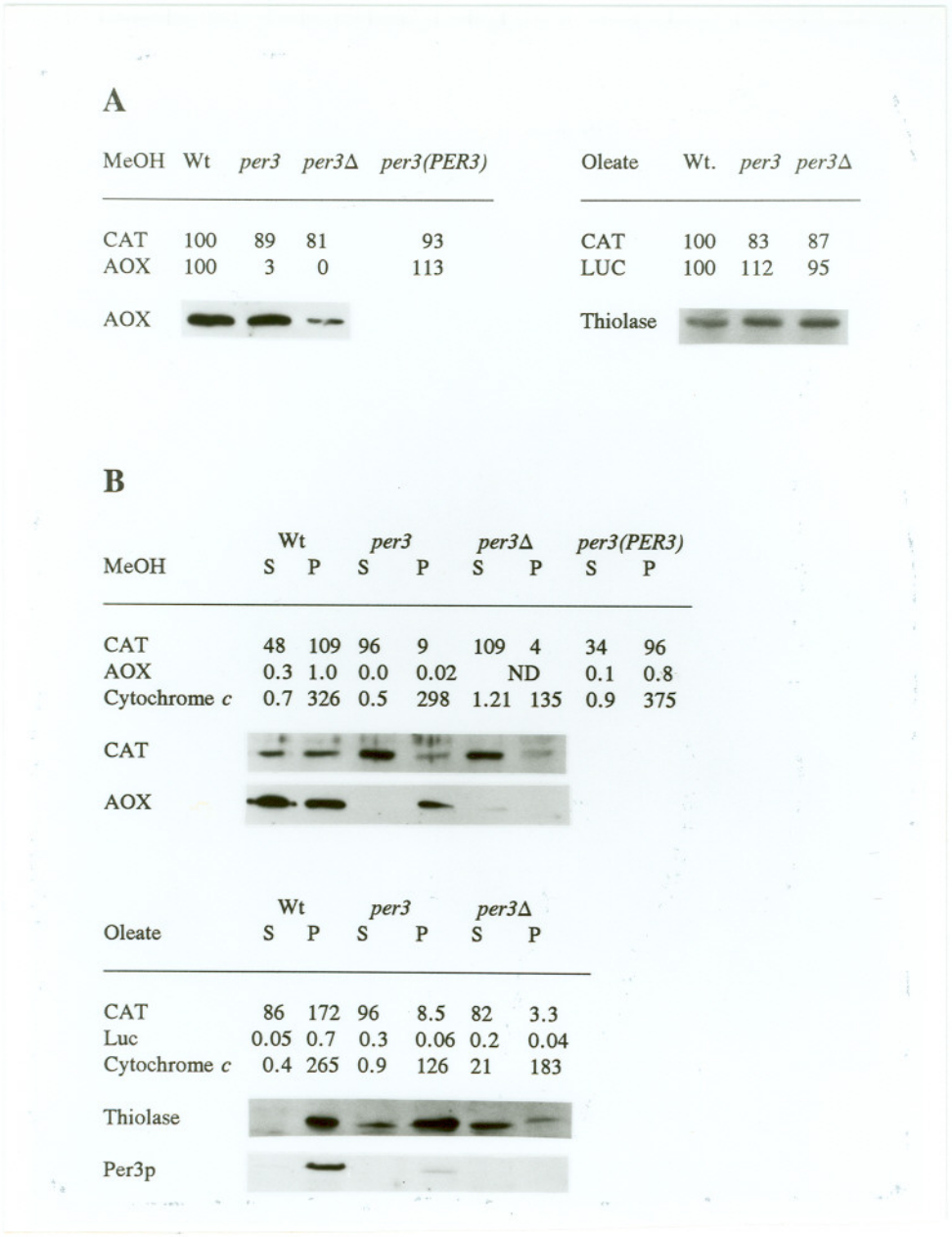

Figure 3.2. Activity and location of selected peroxisomal enzymes in *per3* strains. A, AOX and CAT activity (and AOX protein) levels in methanol-induced strains, and luciferase (*LUC*) and CAT activity (and thiolase protein) levels in oleate-induced strains reported as a percentate of that in wild-type cells. *Wt* , wild-type *P. pastoris*; *per3* , *per3-1*; *per3* (*PER3*), *per3-1* strain JC120 (*per3-1 his4*) transformed with *PER3*-containing plasmid pYT4. B, Specific activity (or relative concentration of protein) in post-20,000 x *g* organelle supernatant (*S*) and pellet (*P*) after differential centrifugation of homogenized protoplasts. ND, could not be determined due to absence of AOX activity. In immunoblots, each lane contains 10 µg of protein. Activity units are as follows: CAT, ΔE_{240}/min/mg of protein; AOX, micromoles of product/min/mg; cytochrome c oxidase, micromoles of product/min/mg x 10^{-3}; LUC, arbitrary light units/mg of protein.

3.4.2 *per3-1* Cells are Defective in Import of Luciferase

Although it is assumed that CAT, DHAS and AOX are all PTS1 enzymes, this has not been directly demonstrated in *P. pastoris*. As direct evidence that *per3-1* was defective in import of PTS1 proteins, we examined the behavior of heterologously expressed luciferase, the prototypical PTS1 enzyme (Gould et al., 1987). Wild-type and *per3-1* strains were constructed that contained luciferase under control of the *AOX1* promoter of *P. pastoris*. Both strains synthesized high levels of luciferase activity in methanol medium, moderate levels in oleate medium, and no luciferase in glucose medium (Fig. 3.2A) (McCollum et al., 1993). After differential centrifugation of preparations from oleate-induced cells of these strains, luciferase activity was located primarily in the pellet fraction from wild-type cells while activity in *per3-1* cells was soluble and present in the supernatant fraction (Fig. 3.2B). From this, we concluded that the *per3-1* strain was defective in import of luciferase and probably other PTS1-containing proteins as well.

3.4.3 *per3-1* Cells Import Thiolase into Peroxisome Remnants

The fate of thiolase, a peroxisomal ß-oxidation enzyme imported via the PTS2 pathway, was also examined in *per3-1* cells (Swinkels et al., 1991; McCollum et al., 1993; Glover et al., 1994b). Crude organelle pellet and supernatant fractions from oleate-induced cells were immunoblotted and reacted with antibodies against *S. cerevisiae* thiolase (Fig. 3.2B). In contrast to CAT, AOX, DHAS, and luciferase, thiolase was found mostly in the pellet, suggesting that this PTS2 enzyme had either aggregated or was located in a subcellular compartment. To distinguish between these possibilities, the location of thiolase was examined immunocytochemically. In sections from oleate-grown wild-type cells, peroxisomes were specifically labeled by antibodies directed

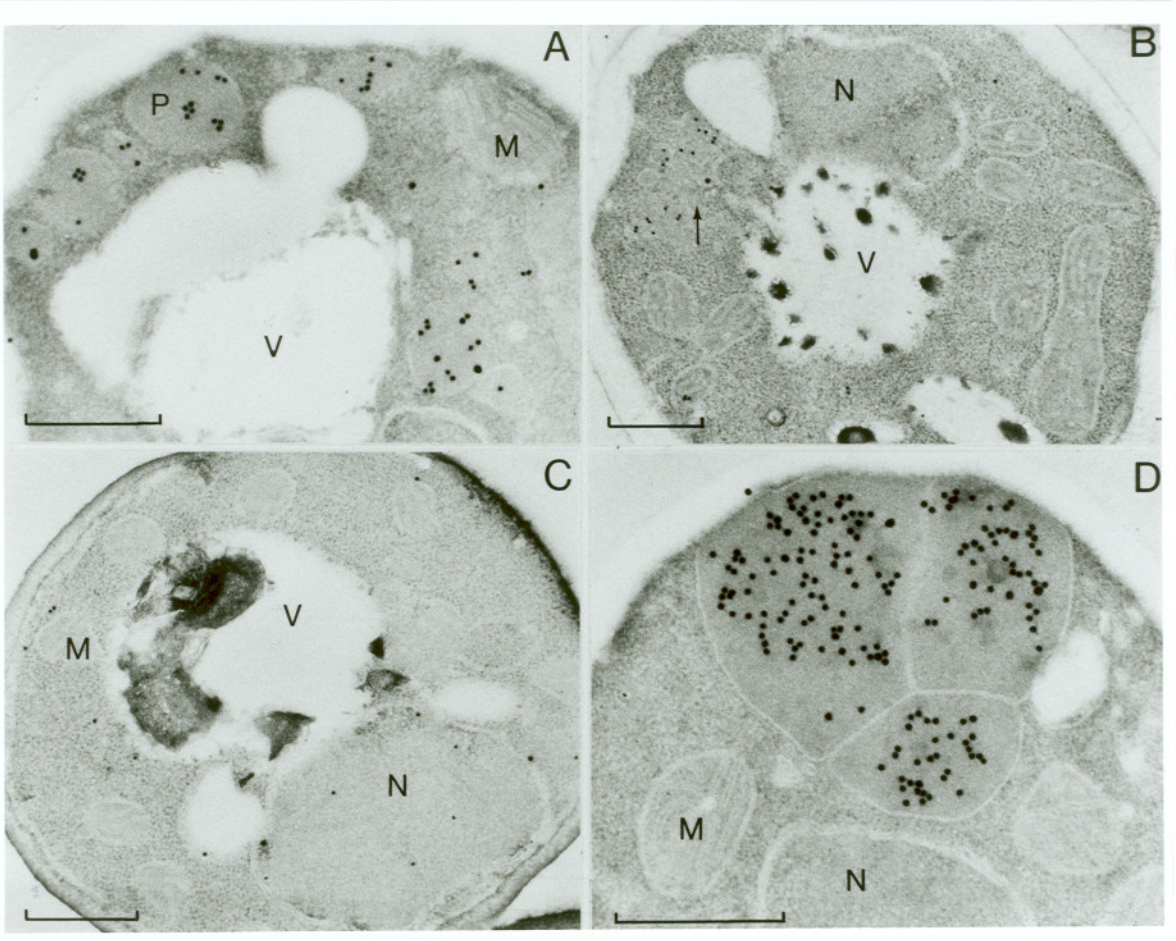

Figure 3.3. Immunocytochemical labelling of oleate-induced celles with anti-thiolase antibodies. Thiolase labelling is concentrated in peroxisomes in wild-type cells (A) and in vesicles in *per3-1* cells (B), but is located in cytosol and nucleus in per3Δ cells (C). Methanol-grown cells of the *per3-1* strain complemented with *PER3*-containing plasmid pYT4 contain normal peroxisomes with AOX protein in the organelles (D). Bar, 0.5 μm.

against *C. tropicalis* thiolase (Fig. 3.3A). In oleate-induced *per3-1* cells, the gold particles were primarily observed on the vesicular structures (Fig. 3.3B), indicating that thiolase was located in these structures. Taken together, our results suggested that *per3-1* was specifically impaired in ability to import CAT, AOX, DHAS and luciferase (PTS1 enzymes) but not thiolase (a PTS2 enzyme) which appeared to be efficiently imported. Furthermore, the presence of thiolase and other peroxisomal enzymes in the vesicular structures indicates that these structures in fact represent peroxisomal remnants.

3.4.4 Isolation and Characterization of *PER3*

The *PER3* gene was isolated from a *P. pastoris* genomic DNA library by functional complementation of *per3-1* strain JC120 (*per3-1 his4*). Library transformants were initially selected for histidine prototrophy (His^+) on glucose medium and subsequently selected for growth on methanol (Mut^+). Total DNA was extracted from a pool of His^+ Mut^+ transformants and plasmids were recovered by transforming the DNA into *E. coli*. A plasmid, designated pYT4 (Fig. 3.4), was isolated that co-transformed the *per3-1 his4* strain to both His^+ and Mut^+ simultaneously. *per3-1* cells transformed with pYT4 contained normal-appearing peroxisomes (Fig. 3.3D). In addition, AOX activity in methanol-grown transformants was restored to normal levels and both AOX and CAT were again present in peroxisomes (Fig. 3.2 and 3.3D). Restriction mapping of pYT4 revealed a *P. pastoris* DNA insert of approximately 8.1 kb. By subcloning selected subfragments from pYT4, the complementing activity was located within a region of 5.0 kb. Northern blots using selected fragments from this region revealed a transcript of approximately 2.1 kb that was present at low levels in glucose-grown wild-type *P. pastoris* and induced approximately five-fold in methanol- or oleate-grown cells (Fig. 3.5). Since peroxiosomes are also induced by these substrates, the higher level of this

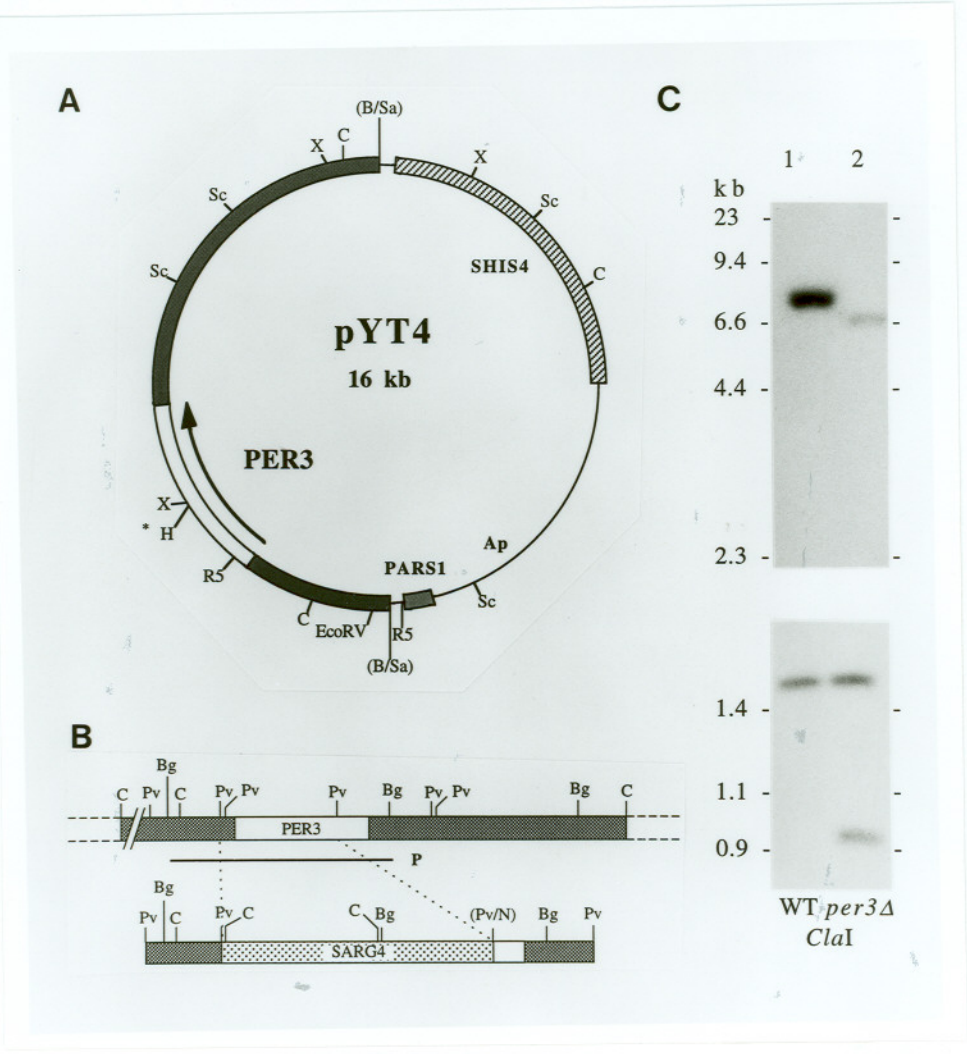

Figure 3.4. Diagrams of plasmid pYT4 and the *PER3*-deletion allele *per3Δ*. A, restriction map of the *PER3* locus pYT4. The *asterisk* marks a *Hind*III site in the *PER3* gene that was used for subcloning but is not unique to the vector. B, the structure of the *per3Δ* allele. To construct this allele, *PER3* DNA fragment containing the *S. cerevisiae* genome by homologous recombination. C. correct targeting of the *per3Δ* fragment was demonstrated by Southern blotting of genomic DNAs cut with *Cla*I and hybridizing with a labeled fragment (P) indicated in B. Lane 1, 2 µg of wild-type genomic DNA; lane 2, 2 µg of DNA from the *per3Δ* strain *JC*121.

transcript was consistent with it being the product of a peroxisomal gene. The DNA sequence of the regions revealed an opening frame (ORF) of 2,139 bp with the potential of encoding a polypeptide of 713 amino acids (~81 kDa) (Fig. 3.6A). Further studies indicated that this ORF was *PER3*. As described below, a *P. pastoris* strain in which most of the ORF was deleted was peroxisome deficient and was determined genetically to be an allele of *per3-1*. In addition, the product of the ORF was found to be a peroxisomal protein.

Hydropathy analysis indicated that the predicted PER3 product (Per3p) was hydrophobic in overall character with at least three potential α-helical transmembrane domains (Fig. 3.6B, residues 338-358, 373-392 and 637-660 in 3.6A) (Kyte and Doolittle, 1982). Two additional regions with lower but significant transmembrane potential were also identified (Fig. 3.6A, residues 484-510 and 522-540). The primary sequence of Per3p showed significant similarity to that of Per1p, a protein required for peroxisome biogenesis protein in *H. polymorpha* (Waterham et al., 1994) (see Discussion). Database searches revealed no other proteins with overall sequence similarity to Per3p. The only identifiable sequence feature of Per3p was the three carboxyl-terminal amino acids (Ala-Lys-Leu-COOH), a known PTS1 sequence variant.

3.4.5 Per3p is a Peroxisomal Membrane Protein

Per3p was characterized through rabbit polyclonal antibodies raised against Per3p expressed in *E. coli* as a fusion with maltose binding protein. In immunoblots prepared from methanol- or oleate-grown wild-type extracts, affinity-purified anti-Per3p antibody preparations recognized an approximately 76-kDa polypeptide that was not present in extracts from methanol- or oleate-induced cells of a strain in which most of *PER3* had been deleted (*per3Δ*) (Fig. 3.2B). The apparent molecular weight of Per3p was somewhat

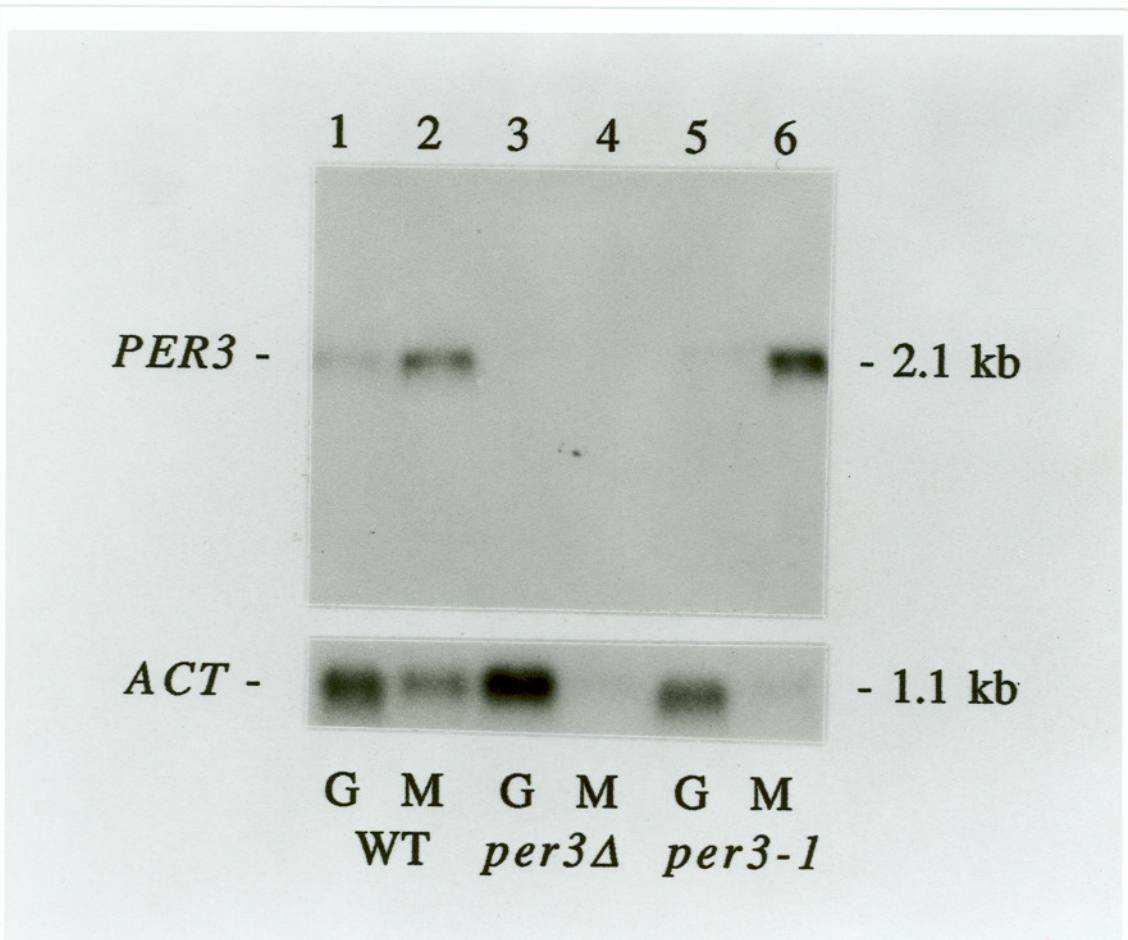

Figure 3.5. Northern blot of *PER3* message. All lanes contain 10 µg of total RNA. *Odd-numbered lanes* contains RNAs from glucose (G)-grown cells. *Even-numbered lanes* contain RNAs from methanol (M)-induced cells. Lanes 1 and 2, wild-type *P. pastoris*; lanes 3 and 4, *per3Δ*; lanes 5 and 6, *per3-1*. As a control, the filter was also bybridized with a labeled DNA fragment encoding the *S. cerevisiae* actin gene (*ACT*). *ACT* message levels are significantly lower in methanol-grown wild-type *P.pastoris* cells than in glucose-grown cells. Levels are even lower in methanol induced cells of the *per3* mutants.

less than the 81-kDa weight calculated from the predicted primary sequence. This discrepancy could be due to the high content of non-polar amino acids in Per3p(42%) which can cause proteins to migrate faster in SDS-PAGE (Büchel et al., 1980). Per3p was present at a significantly higher level in methanol- and oleate-grown cells than in glucose-grown cells (not shown), a result that was consistent with our observations of *PER3* mRNA and the induction of peroxisomes under these growth conditions.

As a first step in establishing the subcellular location of Per3p, methanol- and oleate-grown wild-type *P. pastoris* cells were subjected to differential centrifugation as described above and the resulting pellet and supernatant fractions were examined for Per3p by immunoblotting. Per3p was present primarily in the organelle pellet along with a large portion of CAT (Fig. 3.2B). A crude organellar pellet from oleate-grown cells was then layered over a discontinuous sucrose gradient and centrifuged to separate peroxisomes from other organelles (mainly mitochondria). In these gradients, peroxisomes were concentrated at a density of 1.21 g/cm^3 as judged by the location of CAT and acyl-CoA oxidase activities, and thiolase protein (Fig. 3.7A, fraction 6). Mitochondria were located at a density of 1.16 g/cm^3 as judged by fumarase activity (Fig. 3.7A, fraction 12). Per3p was found exclusively in the peroxisome fraction, indicating that the protein is peroxisomal. Samples of purified peroxisomes from these fractions were extracted with either triethanolamine or sodium carbonate. Following triethanolamine treatment and centrifugation at 30,000 x g, CAT and thiolase were located mostly in the supernatant, whereas Per3p was in the pellet, suggesting that Per3p was associated with the peroxisomal membrane (Fig. 3.7B, lanes 2 and 3). After carbonate extraction and centrifugation at 200,000 x g, most Per3p remained in the membrane pellet, suggesting that Per3p may be an integral peroxisomal membrane protein (Fig. 3.7B, lanes 4 and 5). However, we repeated the carbonate treatment using

A

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-79  cgacccgtataaatagcctctgcgtcctcagtcaacaagacgaagcaatttttttttacctatcttcaggtgcctgtta

  1  ATG TAT AGA TTG GGA TCT CAG GGA AGG TCG ATA CAA AGC CAG CTT CAG AAT GGC GAC TCA
  1    M   Y   R   L   G   S   Q   G   R   S   I   Q   S   Q   L   Q   N   G   D   S

 61  AGT TCG GGA CGA CCA CTA CAG TTG CAA GGA ACT GGG ATG AGA GAA GCT CAG AGG ATT CCT
 21    S   S   G   R   P   L   Q   L   Q   G   T   G   M   R   E   A   Q   R   I   P

121  CAG CAG TTG GAT TAT CTT TTG GCG GAG ATT ATC TCT CCT AAT GAA GAC ACC AAT GTT ATT
 41    Q   Q   L   D   Y   L   L   A   E   I   I   S   P   N   E   D   T   N   V   I

181  GGA TAT CTA GCT TAT TAC TAT CCG AAA CTG AAG AAT GAA CAG AAC GTG GCA CTT TTG ACT
 61    G   Y   L   A   Y   Y   Y   P   K   L   K   N   E   Q   N   V   A   L   L   T

241  GAT TTT TTT CTT AGA TGC CCA ACC TAC TTT AGT CAT TCA AAT GTA GTA TCT CTG AGA AAC
 81    D   F   F   L   R   C   P   T   Y   F   S   H   S   N   V   V   S   L   R   N

301  AAT TAC CCA GTG ATG GAA GCC TTC AAT TAC ATT ATG ACT ACA AAG TTC AAA GTC TCC CAA
101    N   Y   P   V   M   E   A   F   N   Y   I   M   T   T   K   F   K   V   S   Q

361  CCA ACG GTT CCC TTC TAT AGA TTC TAT GCT GCT GTG CTG GCA AGT CTA CTG AAC TGC GAA
121    P   T   V   P   F   Y   R   F   Y   A   A   V   L   A   S   L   L   N   C   E

421  AAG ACC GAT CCA TCG CAC CAT TGG AAG CTG ATT CCA ATA CTA ACT GGA GTC CTT CTC AGC
141    K   T   D   P   S   H   H   W   K   L   I   P   I   L   T   G   V   L   L   S

481  ATC AAA GGA AGA GAT GAC GTT GAG CTA TAT CCC GAT CAT AGC AGG TCT ATC AAG GGA AGT
161    I   K   G   R   D   D   V   E   L   Y   P   D   H   S   R   S   I   K   G   S

541  GAC ACT GCA GTG GCC CAA CTG TTA CAG AGA TGT TTA TTA AGA TTC TAC CAA TCC GGT GAT
181    D   T   A   V   A   Q   L   L   Q   R   C   L   L   R   F   Y   Q   S   G   D

601  GCA AGA AGT TAT GAT TTG AAT GCC CTC GTC ATA ATA TCC ATG AGC TGT GCC TTG GAT TAC
201    A   R   S   Y   D   L   N   A   L   V   I   I   S   M   S   C   A   L   D   Y

661  GTT GAA GAT GAT ACC ATC AAG AAA ATA CTG TAT TGT TTC AAT TAT ACA AGA GCT ATC ATT
221    V   E   D   D   T   I   K   K   I   L   Y   C   F   N   Y   T   R   A   I   I

721  GAT CTT ATT TAT TAT TCT CCT TAT GGT CTC AAT GAT AGT GAC ATA CCG CTA TTG TCG GAC
241    D   L   I   Y   Y   S   P   Y   G   L   N   D   S   D   I   P   L   L   S   D

781  TCT TCC GTT AAC TCA CAG TCT TTC GAC CAG CTA CTT AAC AAC AAC CCC GCG CTG AAA CAT
261    S   S   V   N   S   Q   S   F   D   Q   L   L   N   N   N   P   A   L   K   H

841  CTG AAC CGA TTA TCG TTC CTT TTT GAA AGG ACT GTA AAG TTG AAC GAT GGT AGT ATT CAA
281    L   N   R   L   S   F   L   F   E   R   T   V   K   L   N   D   G   S   I   Q

901  TCA AAT CTT AAT GAT ATT GAT ATT TCC TTG AAC AAG ATG CAA AGC TTC AGC GAG AAA CTT
301    S   N   L   N   D   I   D   I   S   L   N   K   M   Q   S   F   S   E   K   L

961  TCA AAG AAA ATT TCC GTT CTA GAT GAT GAT AGT AGC AAA GGG GTT GGC CAG CTT CTC AGA
321    S   K   K   I   S   V   L   D   D   D   S   S   K   G   V   G   Q   L   L   R

1021 CAG TGT TTA TAT GCT TCA ATC ATC ATT CAT CAA GCT ATC CTG ACG ACG TTT TTC CAG TTG
341    Q   C   L   Y   A   S   I   I   I   H   Q   A   I   L   T   T   F   F   Q   L

1081 GAT AAT GCA GAC TAT ACA AAG TAT TTT TTG CCG TCA TTT TCC AGA AAG ATT CTT TCC ATT
361    D   N   A   D   Y   T   K   Y   F   L   P   S   F   S   R   K   I   L   S   I

1141 TTA TTT AAC TTA TTC TTC ATT GTG GAC AGA ATT GGA ACG GGT GGA TTC CAG CCC TAC AAC
381    L   F   N   L   F   F   I   V   D   R   I   G   T   G   G   F   Q   P   Y   N

1201 TTT GTG TAT TTA ACA TGC TTG CAA GGG ATA ATT CAG TAC GAC ATG AAA ACT GCC GAA TCA
401    F   V   Y   L   T   C   L   Q   G   I   I   Q   Y   D   M   K   T   A   E   S

1261 CTT GTG AAG ACA TTT ACC ACT GGC ATT AAC TAC TCA TCT TTG AAA GAT TCT GAG GTT GCA
421    L   V   K   T   F   T   T   G   I   N   Y   S   S   L   K   D   S   E   V   A

1321 AGG GCT AAA CTT TTG TTC ACT CTC AAT TTG ATG GAA CAG ATT GTG AAT ATT TGC AGC GAT
441    R   A   K   L   L   F   T   L   N   L   M   E   Q   I   V   N   I   C   S   D

1381 GAT CTA AGA TTG GAA CTA ATC GTA CCG TTG GTA GAA GAT TTG GTG AAC AAC AAA AAT GCA
461    D   L   R   L   E   L   I   V   P   L   V   E   D   L   V   N   N   K   N   A
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1441  TGT GTT GAT ATT CAT AAT CAT GTG TTC AAG AGT ATA TTT GAA TCT GCT CAT TCA GTT ATC
 481    C   V   D   I   H   N   H   V   F   K   S   I   F   E   S   A   H   S   V   I

1501  CTT AAG TTT TTT ACT GTG GTG GAC TCT TCC GTA AAG AAT GTT GAC TAT GAG ACA AAT GTT
 501    L   K   F   F   T   V   V   D   S   S   V   K   N   V   D   Y   E   T   N   V

1561  ACA TTA GTA TCA GAA AAG ATC ATC CCT TAT TTG ACG CTT GTC ATT GAT CAG TTT CCG GAA
 501    T   L   V   S   E   K   I   I   P   Y   L   T   L   V   I   D   Q   F   P   E

1621  TTT TTA TCA ATT AAT CAG CTG GAT ATT GCA ATT GAG ACT ATT AGT CGT ACC GTA TTT CCA
 541    F   L   S   I   N   Q   L   D   I   A   I   E   T   I   S   R   T   V   F   P

1681  GAC AGT CCC ATC TAC AGT TAC GAC AAG AAC ATC TCA TCT ATG TTT TTG AAC GTC TTG TTT
 561    D   S   P   I   Y   S   Y   D   K   N   I   S   S   M   F   L   N   V   L   F

1741  AAC AAA TGT TTA ACC GTT GAC AAC GAT GAG TTA GTT GAG TTG CCC GCA ATC GAG GCA GTA
 581    N   K   C   L   T   V   D   N   D   E   L   V   E   L   P   A   I   E   A   V

1801  GTT GCA CCT AAG AAC GAC GAA GAA AAT AAT ACG TCT GAC GCG CAA GAT GGC GGA CCT AAA
 601    V   A   P   K   N   D   E   E   N   N   T   S   D   A   Q   D   G   G   P   K

1861  GAA CTT CAG TCA TTG AAT GAT TTA AAA TCC AGA AGA TCG GCA TTG ATT TCT GCT TTA ATC
 621    E   L   Q   S   L   N   D   L   K   S   R   R   S   A   L   I   S   A   L   I

1921  TCA GTT TTC CCT CTT ATT CCC GTG AAG GAC TAC ACT AAA TGG TTG TCA ATT GCT TTC TAC
 641    S   V   F   P   L   I   P   V   K   D   Y   T   K   W   L   S   I   A   F   Y

1981  GAC TTG ATT GTA GCT ACC CCA GAA AGA ACG GAA CGT GCA TTT TTG CAA GAA CGA TTG TGG
 661    D   L   I   V   A   T   P   E   R   T   E   R   A   F   L   Q   E   R   L   W

2041  GAC TGC GTT GTT GGA ACC AAC AAA TAT GAC CCT CAA AAG GGA AAT TTA GGT ATA ATG TGG
 681    D   C   V   V   G   T   N   K   Y   D   P   Q   K   G   N   L   G   I   M   W

2101  TGG TAT GAG AAT GTA AAC GCC CAA TCA ACC GCA AAG TTA TAA gatttctgttggatacattgtga
 701    W   Y   E   N   V   N   A   Q   S   T   A   K   L  ***

2166  ttagcccaaactccactgattccattactatatagttgtgtattttattattcgaggtgatgagatagagaaatcatcg
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B

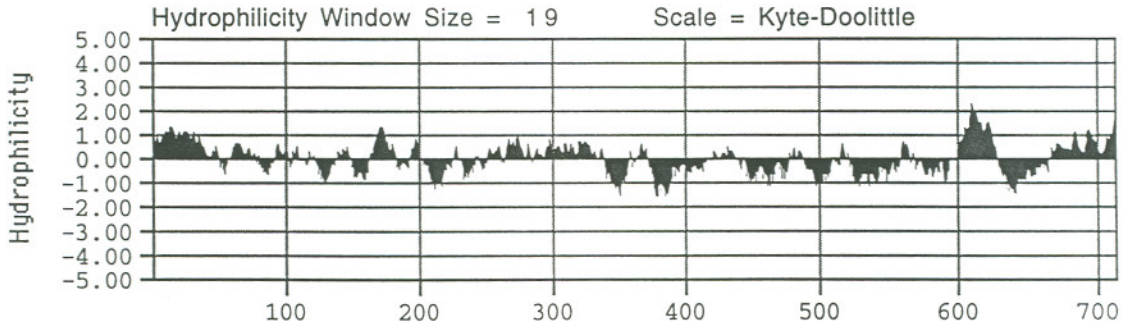


Figure 3.6. *PER3* sequence. A, nucleotide and preicted amino acid sequences of *PER3*. Potential α-helical membrane-spanning domains are *underlined.* B. hydrophilicity plot of Per3p-predicted primary structure shows that the peptide is hydrophobic in overall character. These sequence data are available from EMBL/GeneBank™/DDJB under acccession no. L40485.

crude organelle pellet fractions from methanol- and oleate-induced cells and found that, in contrast to results with purified peroxisomes, only about 25 % of Per3p was resistant to extraction (data not shown). Similar results have been obtained with the *S. cerevisiae* 24-kDa peroxisomal membrane protein (Pmp24p) where the inextractibility of the protein from purified peroxisomes was believed to be an artifact due to the extensive manipulations involved in the isolation of peroxisomes (J. Goodman, personal communication). We conclude that Per3p is tightly associated with the peroxisomal membrane but is probably not an integral membrane protein.

3.4.6 A *PER3*-Deletion Strain Lacks Peroxisomes and Contains Cytosolic Thiolase

A *P. pastoris* strain in which most of *PER3* was deleted was created by the gene replacement method (Rothstein, 1983). For the replacement, a plasmid were constructed in which 1638 bp of *PER3* coding sequence (nucleotides 1 through 1638 encoding amino acids 1 through 546 in Fig. 3.6A) was removed and replaced with a fragment containing the *S. cerevisiae ARG4* gene. This plasmid was then digested with a restriction enzyme to release the *PER3* deletion allele (*per3Δ*) on the linear DNA fragment shown in Fig. 3.4B, and introduced into *P. pastoris* strain GS190 (*arg4*). Transformants in which the fragment had deleted the *PER3* locus were isolated by selecting for Arg^+ colonies and then screening for ones which were also Mut^-. Proper targeting of the fragment was confirmed by Southern blot analysis (Fig. 3.4C). In addition, northern blots showed that methanol-induced cells of *per3Δ* strains no longer produced the 2.1-kb *PER3* message (Fig. 3.5B, lanes 3 and 4). One *per3Δ*-derived strain, JC121 (*per3Δ::SARG4 arg4*), was crossed with JC120 (*per3-1 his4*) by selection for growth on minimal glucose plates. The resulting diploids were tested for methanol growth and were Mut^-. Several thousand spore products from these diploids were then examined by replica plating for Mut phenotype and all were Mut^- as well, demonstrating that the *per3-1* and *per3Δ* alleles

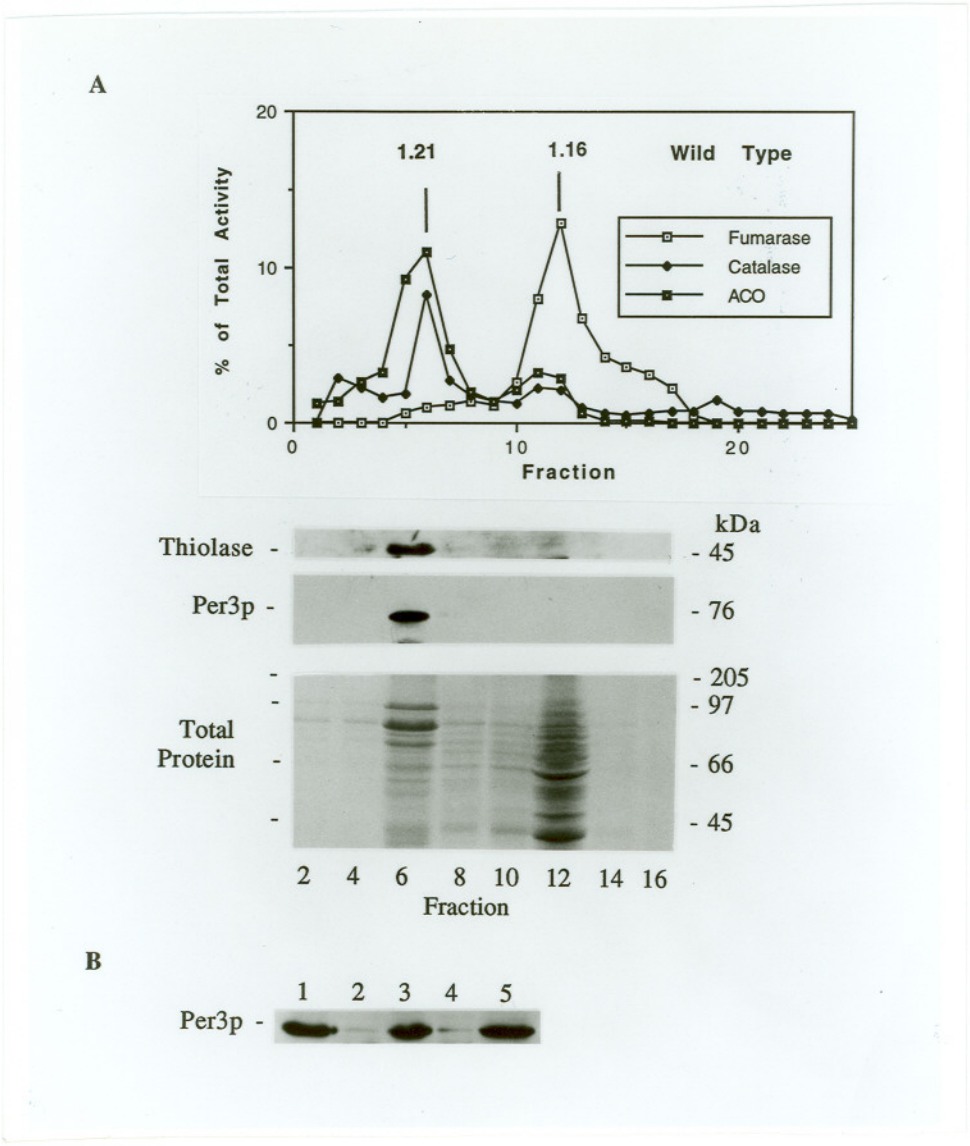


Figure 3.7. Per3p is a peroxisomal membrane protein. A. sucrose density profile of crude organelle pellet fraction derived from oleate-induced wild-type *P.pastoris* cells. Activity in each fraction is presented as a percentage of total activity in gradient. *Fraction 1 (left)* is gradient bottom and *fraction 25 (rightI) is gradient top. ACO*, acyl-CoA oxidase activity. Immunoblotting was performed with equal volumes (200 µl) of selected gradient fractions. Total protein was visualized by staining with Coomassie Blue dye. B. In lanes 1 through 5, 25 µg of purified peroxisomes isolated from oleate-grown wild-type cells (lane 1) were extracted with 20 mM triethanolamine, pH 7.8 (lantes 2 and 3) or 0.1 M sodium carbonate, pH 11 (lanes 4 and 5). After extraction, samples were centrifuged, and supernatant (*even-numbered lanes*) and pellet (*odd-numbered lanes*) fractions were immuunoblotted with anti-Per3p antibodies.

were tightly linked and likely to be mutant alleles of the same gene.

Phenotypically, the *per3Δ* strain was clearly a *per* mutant. In addition to being Mut$^-$ and Out$^-$, EM examination of methanol-induced *per3Δ* cells showed that they lacked intact peroxisomes, and biochemical studies showed that CAT was induced to normal levels in both methanol- and oleate-induced cells but located in the cytosol (Fig. 3.2). However, the *per3-1* and *per3Δ* strains were different in several respects. First, methanol-induced cells of *per3-1* frequently contained clusters of peroxisomal remnants, which were absent in methanol- or oleate-induced *per3Δ* cells (Fig. 3.1E). Thus, the phenotype of a *per3* null mutant strain appears to be the complete absence of peroxisomes. Second, methanol-induced *per3Δ* cells contained no AOX activity whereas *per3-1* cells contained low but significant amounts of sedimentable AOX activity (Fig. 3.2A, B). These results support our suggestion that the *per3-1* allele in the mutant strain is slightly leaky with respect to AOX import and that active AOX enzyme is solely present in peroxisomal remnants.

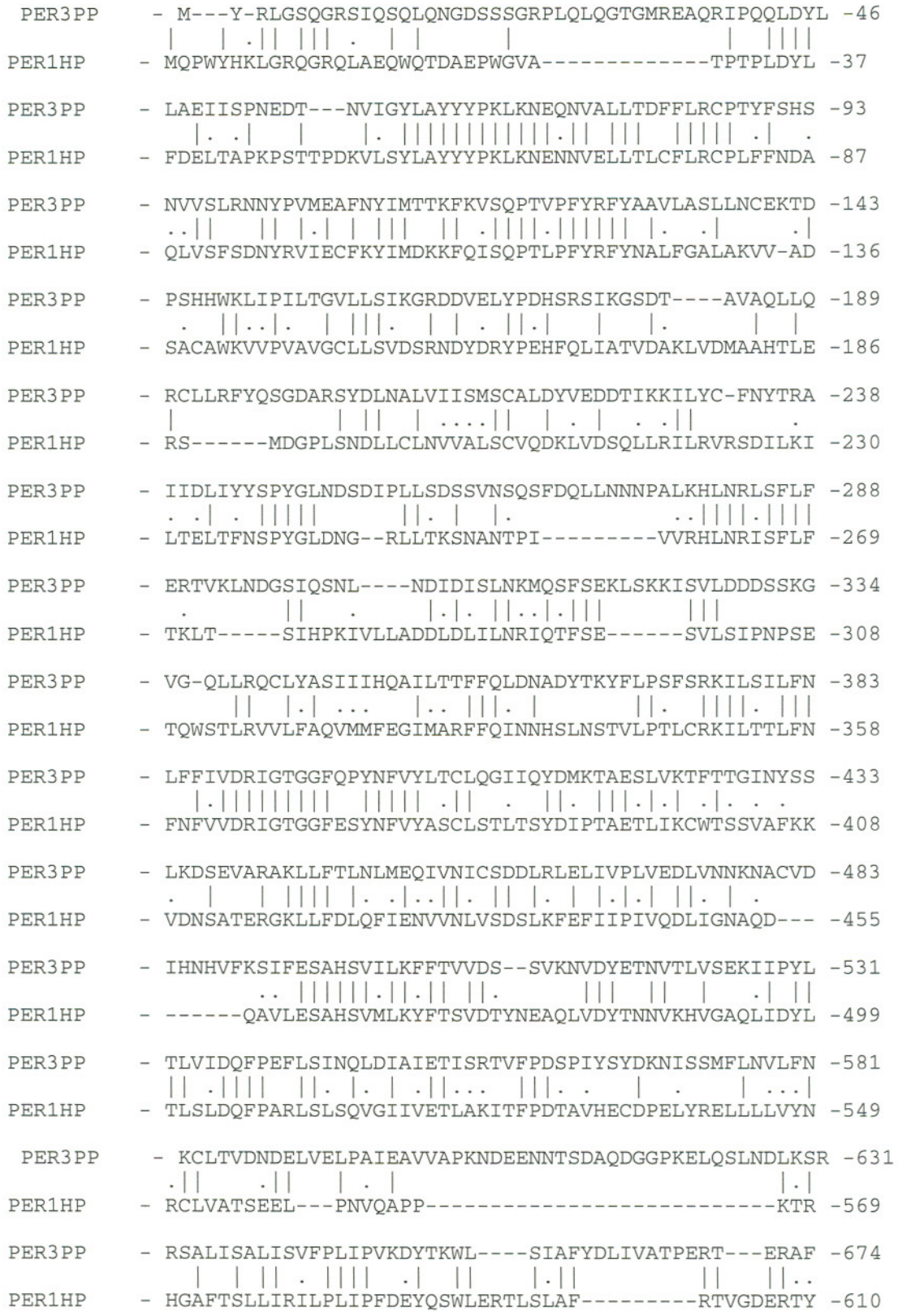
Third, biochemically (Fig. 3.2B) and immunocytochemically (Fig. 3.3C) thiolase was found to be located in the cytosol. These results were in contrast to induced *per3-1* cells where thiolase was located primarily within the peroxisomal remnants (Figs. 3.2B, 3.3B). Thus, although both *per3-1* and *per3Δ* were deficient in ability to import CAT, AOX and luciferase, *per3-1* cells selectively imported thiolase while *per3Δ* cells did not.

3.5 Discussion

Previously we described the isolation and partial characterization of a collection of *P. pastoris* mutants defective in peroxisome biogenesis (*per* mutants) (Liu et al., 1992; Gould et al., 1992). In this study, we report the detailed analysis of mutants in *PER3* as well as the isolation and characterization of *PER3* and its product Per3p. The primary sequence of Per3p predicts a 713 amino acid protein that is hydrophobic in overall character with two potential α-helical transmembrane domains. Consistent with these predictions, biochemical studies showed that Per3p has an apparent molecular mass of approximately 76 kDa and is tightly associated with the peroxisomal membrane and may even be an integral membrane protein of the organelle. Per3p terminates in the tripeptide sequence Ala-Lys-Leu-COOH (AKL), a variant within the PTS1 group that is sufficient to target reporter proteins to mammalian peroxisomes (Swinkels et al., 1992; Waterham et al., 1994). The presence of a PTS1 on Per3p is surprising since this targeting signal is thought to be responsible for import of matrix proteins and not membrane proteins (de Hoop and AB, 1992). Other peroxisomal proteins that specifically end in AKL include: mammalian sterol carrier 2 (Yamamoto et al., 1991), glycosomal glyceraldehyde phosphate dehydrogenase of *Trypanosoma brucei* (Michels et al., 1986), acetoacetyl-CoA thiolase A of *Candida tropicalis* (Kurihara et al., 1992), PMP20 of *Candida boidinii* (Garrard and Goodman, 1989), and Per1p of *Hansenula polymorpha* (Waterham et al., 1994). However, only a few peroxisomal membrane proteins have been described and it is not yet clear whether PTS1 participates in targeting of some membrane proteins. It will be interesting to determine whether the motif on Per3p is a functional targeting signal and whether it is responsible for targeting Per3p to the peroxisomal membrane.

Database searches revealed only one other protein with significant sequence similarity to Per3p. Per1p, a protein required for peroxisome biogenesis in the methylotrophic yeast *H. polymorpha,* is 42% identical and 60% similar to Per3p and may be functional homologues (Fig. 3.8) (Waterham et al., 1994). Despite their similarity, interesting differences between the proteins are evident. First, Per1p is significantly smaller than Per3p (~71 kDa versus 81 kDa) and contains a functional PTS2 sequence not present in Per3p. Second, *P. pastoris* and *H. polymorpha* are closely related species (Kurtzman, 1984) and other proteins show a much greater degree of similarity between these two yeast species. For example, the primary sequences for AOX and DHAS are each greater than 80% identical (Janowicz et al., 1985; Ledeboer et al., 1985; Koutz et al., 1989; G. Thill, personal communication). Thus, it might be expected that the *H. polymorpha* homologue of Per3p would show greater similarity than Per1p. Third, Per3p is a membrane protein while Per1p is located in the peroxisomal matrix (Waterham et al., 1994). Fourth, the *H. polymorpha PER1* gene does not complement *per3* mutants of *P. pastoris* and *PER3* does not complement *H. polymorpha per1* (J. Cregg, unpublished results). In contrast, the *H. polymorpha* genes for *AOX* and formaldehyde dehydrogenase (another methanol-pathway specific enzyme) readily complemented *P. pastoris* mutants in these genes and northern blots indicated that transcription of the *H. polymorpha* genes initiates correctly and is properly regulated (J. Cregg, unpublished results). Thus, it is possible that Per3p and Per1p are not homologues but are different members of a family of related proteins required for peroxisome biogenesis.

Our studies of *per3-1*, a nitrosoguanidine-generated mutant, show that the numerous membranous vesicles frequently observed in induced cells of this strain are peroxisomal in origin. In oleate-induced *per3-1* cells these vesicular structures contain thiolase but only minor amounts of PTS1 proteins while the bulk of these enzymes are present in the


PER3PP - M---Y-RLGSQGRSIQSQLQNGDSSSGRPLQLQGTGMREAQRIPQQLDYL -46
PER1HP - MQPWYHKLGRQGRQLAEQWQTDAEPWGVA----------TPTPLDYL -37
PER3PP - LAEIISPNEDT---NVIGYLAYYYPKLKNEQNVALLTDFFLRCPTYFSHS -93
PER1HP - FDELTAPKPSTTPDKVLSYLAYYYPKLKNENNVELLTLCFLRCPLFFNDA -87
PER3PP - NVVSLRNNYPVMEAFNYIMTTKFKVSQPTVPFYRFYAAVLASLLNCEKTD -143
PER1HP - QLVSFSDNYRVIECFKYIMDKKFQISQPTLPFYRFYNALFGALAKVV-AD -136
PER3PP - PSHHWKLIPILTGVLLSIKGRDDVELYPDHSRSIKGSDT----AVAQLLQ -189
PER1HP - SACAWKVVPVAVGCLLSVDSRNDYDRYPEHFQLIATVDAKLVDMAAHTLE -186
PER3PP - RCLLRFYQSGDARSYDLNALVIISMSCALDYVEDDTIKKILYC-FNYTRA -238
PER1HP - RS------MDGPLSNDLLCLNVVALSCVQDKLVDSQLLRILRVRSDILKI -230
PER3PP - IIDLIYYSPYGLNDSDIPLLSDSSVNSQSFDQLLNNNPALKHLNRLSFLF -288
PER1HP - LTELTFNSPYGLDNG--RLLTKSNANTPI---------VVRHLNRISFLF -269
PER3PP - ERTVKLNDGSIQSNL----NDIDISLNKMQSFSEKLSKKISVLDDDSSKG -334
PER1HP - TKLT-----SIHPKIVLLADDLDLILNRIQTFSE------SVLSIPNPSE -308
PER3PP - VG-QLLRQCLYASIIIHQAILTTFFQLDNADYTKYFLPSFSRKILSILFN -383
PER1HP - TQWSTLRVVLFAQVMMFEGIMARFFQINNHSLNSTVLPTLCRKILTTLFN -358
PER3PP - LFFIVDRIGTGGFQPYNFVYLTCLQGIIQYDMKTAESLVKTFTTGINYSS -433
PER1HP - FNFVVDRIGTGGFESYNFVYASCLSTLTSYDIPTAETLIKCWTSSVAFKK -408
PER3PP - LKDSEVARAKLLFTLNLMEQIVNICSDDLRLELIVPLVEDLVNNKNACVD -483
PER1HP - VDNSATERGKLLFDLQFIENVVNLVSDSLKFEFIIPIVQDLIGNAQD--- -455
PER3PP - IHNHVFKSIFESAHSVILKFFTVVDS--SVKNVDYETNVTLVSEKIIPYL -531
PER1HP - ------QAVLESAHSVMLKYFTSVDTYNEAQLVDYTNNVKHVGAQLIDYL -499
PER3PP - TLVIDQFPEFLSINQLDIAIETISRTVFPDSPIYSYDKNISSMFLNVLFN -581
PER1HP - TLSLDQFPARLSLSQVGIIVETLAKITFPDTAVHECDPELYRELLLLVYN -549
PER3PP - KCLTVDNDELVELPAIEAVVAPKNDEENNTSDAQDGGPKELQSLNDLKSR -631
PER1HP - RCLVATSEEL---PNVQAPP-------------------------KTR -569
PER3PP - RSALISALISVFPLIPVKDYTKWL----SIAFYDLIVATPERT---ERAF -674
PER1HP - HGAFTSLLIRILPLIPFDEYQSWLERTLSLAF---------RTVGDERTY -610

```
PER3PP    - LQERLWDCVVGTNKYDPQKGNLGIMWWYENVN-AQSTAKL -713
              | . ||| ..|||.  |||| .|| |||| || .|  |||
PER1HP    - LLDLLWDSILGTNRHYPQKGYVGIQWWYEHVNESQEKAKL -650


Identity  : 276 ( 42.5%)
Similarity: 111 ( 17.1%)
Number of gaps inserted in PER3PP: 11
Number of gaps inserted in PER1HP: 11
```

Figure 3.8. Comparison of predicted amino acid sequences of *P.pastoris* Per3p and *H. polymorpha* Per1p. Sequences were aligned using PC gene software. The character "|" between sequences indicates residues that are identical. The character "." indicates similar residues. Similar residues are defined as: *A,S,T,D,E; N,Q; R,K; I,L,M,V; F,Y,W.*

cytosol. This is the first direct visual evidence that these structures, which are observed in most *P. pastoris per* mutants, are remnants of peroxisomes in *P. pastoris*. In Zellweger cell lines, similar peroxisomal remnants, called peroxisomal ghosts, are also observed (Santos et al., 1988b). The additional layer(s) of membrane that often surround peroxisomal remnants may be the result of their inclusion within autophagic vacuoles (Dunn, 1994). In methylotrophic yeasts, cells shifted from methanol to other carbon sources are known to rapidly sequester peroxisomes within autophagic structures where they are degraded by vacuolar enzymes (Veenhuis et al., 1983). Furthermore, in Zellweger cells, a significant portion of the peroxisomal ghosts are enclosed within compartments that contain lysosomal proteins, suggesting that they are subject to rapid turnover by autophagy as well (Heikoop et al., 1992).

Our data suggest that Per3p plays an essential role in matrix protein import. This conclusion is based on observation that the peroxisomal remnant structures in induced *per3-1* cells efficiently import thiolase but not AOX, CAT, DHAS or luciferase. Thiolase is imported via a PTS2 pathway in mammals and yeast (Swinkels et al., 1991; Glover et al., 1994b) whereas luciferase is imported via a PTS1 pathway (Gould et al., 1987). Although PTSs for AOX, CAT and DHAS have not been defined in *P. pastoris*, these enzymes have been shown to harbor PTS1 sequences in *H. polymorpha* (Didion and Roggenkamp, 1992; Hansen et al., 1992) and therefore, are likely to be imported via a PTS1 pathway in *P. pastoris* as well. Thus, *per3-1* cells are primarily affected in PTS1 but not PTS2 import. The *per3-1* strain is not be totally defective in PTS1 import since small amounts of CAT and AOX are found in the peroxisomal remnant structures. Interestingly, upon differential centrifugation of methanol-induced *per3-1* preparations, virtually all of the active AOX enzyme is found in the crude organelle pellets, indicating that only AOX which gains entry into a peroxisome assembles into active octameric

enzyme, while the majority of AOX remains in the cytosol as inactive aggregates. That only imported AOX becomes active suggests that AOX assembly may require the presence of one or more peroxisomal factors such as the AOX co-factor FAD or a peroxisomal assembly factor (Evers et al., 1994).

An additional clue to Per3p function came from studies comparing the *per3-1* strain to a strain deleted for most of *PER3* (*per3Δ*). Although neither *per3* mutant can grow on methanol or oleate and both are peroxisome deficient, they display at least two differences. First, in induced *per3Δ* cells, we cannot find the peroxisomal remnant structures that are so prevalent in *per3-1*. Second, thiolase is in the cytosol in oleate-induced *per3Δ* cells whereas it is in peroxisomal remnants in *per3-1* cells. Thus, the *per3* null phenotype appears to be an inability to import either PTS1- or PTS2-containing proteins.

To explain the *per3* mutant phenotypes, we propose that Per3p is an essential component of the cell's machinery for import of both PTS1 and PTS2 (and perhaps all) matrix proteins. To account for the continued ability of *per3-1* cells to import thiolase, we suggest that Per3p is composed of several functional domains and that in the *per3-1* allele, a mutant product is synthesized that is defective in a domain primarily involved in import of PTS1 proteins but not PTS2 proteins. Consistent with partially functional Per3p, we observe that the mutant protein is present in induced *per3-1* cells and that it is the same size as in wild-type cells (Fig. 3.2B). To explain the phenotype of the *per3Δ* strain, we suggest that other domains in Per3p are essential for PTS2 protein import (or both PTS1 and PTS2 import) and therefore, deletion of *PER3* results in a strain that is defective in all matrix protein import.

The identification of two PTSs for peroxisomal protein import has raised the question of whether cells also have two independent peroxisomal translocation pathways

or only have separate PTS receptors but otherwise share most components of a common translocation machinery (McCollum et al., 1993; Zhang et al., 1993b). Despite the large numbers of peroxisome-deficient yeast mutants that have been characterized, only two appear to be selectively defective in a specific import pathway. *P. pastoris pas8* (*S. cerevisiae pas10*; *H. polymorpha per3*) mutants, are specifically defective in PTS1 protein import and the Pas8p has been shown to specifically bind a peptide ending in the PTS1 sequence, Ser-Lys-Leu-COOH (McCollum et al., 1993; van der Leij et al., 1993; M. Veenhuis, unpublished results). Thus, Pas8p appears to be the PTS1 protein receptor. Conversely, *S. cerevisiae pas7* mutants are selectively defective in PTS2 import (Marzioch et al., 1994). Similar *S. cerevisiae* mutants have been reported as *peb5* and *peb1* and may be alleles of *pas10* and *pas7*, respectively (Zhang et al., 1993b). The small number of pathway-specific mutants is most easily explained as a consequence of a shared import pathway. Our model of Per3p function in which the protein is composed of domains preferentially involved in import of different PTS protein classes also appears to be most consistent with a shared import pathway model.

CHAPTER 4.

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Summary of Research

This thesis describes the initiation of a genetic approach toward understanding the molecular mechanisms controlling peroxisome biogenesis using the methylotrophic yeast *Pichia pastoris* as a model system. I described the isolation and characterization of the first *P. pastoris* mutants that are defective in the biogenesis of peroxisomes (*per* mutants). The mutants were readily isolated from mutagenized *P. pastoris* cultures by screening for strains that could not grow on either methanol or oleic acid as a sole carbon and energy source but could still grow on other carbon sources including glucose and ethanol. I identified 25 mutants that met these phenotypic criteria and determined that 23 were *per* mutants. The *per* mutants harbored recessive alleles representing 8 different complementation groups which were named *PER1* through *PER8*. Electron microscopic studies demonstrated that methanol-induced cells of the *per* mutants lack normal peroxisomes, though small single and multi-membranous vesicular structures were often observed. Differential centrifugation studies indicated that catalase was present in the cytosol in methanol- and oleate-induced *per* mutant cells. Interestingly, alcohol oxidase activity and protein levels were abnormally low in all methanol-induced *per* mutants.

The *per* mutants have been utilized to clone several *PER* genes by functional complementation. This thesis described the isolation and characterization of one of these genes *PER3*. The predicted product of PER3 was found to be a novel hydrophobic

protein of 713 amino acids that ends in the PTS1 sequence AKL. Per3p is tightly associated with the peroxisomal membrane but is probably not an integral membrane protein. Since Per3p ends in a matrix protein PTS, I hypothesize that the protein is located on the matrix side of the peroxisomal membrane. Insight into the possible function of Per3p in peroxisomal biogenesis was obtained through studies of *per3* mutants. In induced cells of *per3-1*, a chemically induced mutant, several PTS1 enzymes were located primarily in the cytosol while the PTS2-enzyme thiolase was found mostly in small vesicular structures. Thus, these structures are most likely remnants of peroxisomes and *per3-1* appears to be preferentially affected in the import of PTS1 enzymes. However, in a mutant deleted for most of *PER3* (*per3Δ*), peroxisomal remnants could not be found and thiolase along with PTS1 enzymes were located in the cytosol. From these results, it was hypothesized that Per3p is required for import of peroxisomal matrix proteins and that it is composed of at least two domains; one involved primarily in the import of PTS1 proteins and the other in the import of PTS2 proteins (or both PTS1 and PTS2 proteins).

4.2 Future Directions

Further experiments on the role of Per3p in peroxisome biogenesis will be directed toward the following questions: (1) What is the topology of Per3p on the peroxisomal membrane; what is the targeting signal(s) for directing Per3p to the peroxisome; (3) if Per3p is composed of functional domains, where are these domains; and (4) what proteins interact with Per3p?

4.2.1 Topology of Per3p

Results suggest that Per3p is tightly associated with the peroxisomal membrane (Liu et al., 1995). From the presence of a PTS1 at the carboxy-terminus of Per3p, we speculate that Per3p is imported into the peroxisome like other PTS1-containing matrix proteins and therefore, that Per3p may be located on the matrix side of the peroxisome. This hypothesis can be tested by treating peroxisomes with protease and observing whether Per3p is sensitive to digestion. If Per3p is located inside the organelle, it should be relatively resistant to protease digestion. If, on the other hand, Per3p is present on the outer surface of peroxisomes, the protein should be readily degraded.

4.2.2 Targeting of Per3p to the peroxisome

An unusual feature of Per3p is the presence of AKL at its COOH-terminus. This is a known functional variant of the PTS1 targeting signal for peroxisomal matrix proteins (Liu et al., 1995). To test whether AKL is necessary for targeting of Per3p to peroxisomes, a *P. pastoris* strain will be constructed with a mutant *PER3* gene in which sequences encoding AKL have been deleted. The strain will be examined for growth on methanol and oleate and the localization of Per3p will be determined by differential centrifugation followed by immunoblotting using anti-Per3p antibodies. To show that AKL is a PTS in *P. pastoris*, sequences encoding the tripeptide will be appended to a nonperoxisomal protein such as chloramphenicol acetyltransferase (bCAT) and the localization of the fusion protein determined by differential centrifugation and immunocytochemistry. If these results show that Per3p contains another PTS (i.e., AKL is not necessary for targeting of Per3p), additional *PER3*-deletion and bCAT-*PER3*-fusion constructs will be tested to define the PTS(s) for Per3p.

4.2.3 Functional dissection of Per3p

We hypothesize Per3p may have separate domains involved in import of PTS1 and PTS2 proteins (Swinkels et al., 1992; Liu et al., 1995). To test this idea and define the location of these domains, *P. pastoris* strains with additional mutations in *PER3* will be constructed. The effects of these mutations on peroxisome function (especially the import of thiolase) will be examined and the location of interesting mutations will be determined by DNA sequencing to give a picture of the functional domains. As a first step, the identify of the mutation responsible for the *per3-1* mutant will be determined. New *per3* alleles will be generated by *in vitro* mutagenesis of a *PER3*-containing plasmid and after transformation of our *per3Δ* host, screened for interesting new *per3* mutants. In addition, interesting regions of *PER3* will be mutated by *in vitro* site-directed mutagenesis.

4.2.4 Identification of proteins that interact with Per3p

Per3p is likely to function as part of a multi-protein complex. Genetic studies in *H. polymorpha* indicate that the products of most *PER* genes interact including *PER1*, a possible homologue of the *P. pastoris PER3* gene (Titorenko et al., 1993; Waterham et al., 1994). Proteins that interact with a known protein whose gene has been cloned can be identified by the two-hybrid protein system (Fields and Song 1989). This is a genetic screening strategy that identifies and isolates genes whose products interact. It is based on the *S. cerevisiae* GAL4 protein which is known to be composed of two independent domains, one for DNA binding and the other for transcriptional activation. To search for Per3p interacting proteins, soluble portions of Per3p will be expressed as a fusion with the GAL4 binding domain (GAL4-Per3p). A library of *P. pastoris* DNA with then be cloned into a second vector that will express proteins as fusions with the GAL4 activation domain. The library will be transformed into an *S. cerevisiae* strain that expresses *lacZ*

gene under control of GAL4 upstream activation sequences. Transformants that express ß-galactosidase are identified by their blue color on indicator plates. Positive colonies are candidates for containing a *P. pastoris* gene that interacts with Per3p. The *P. pastoris* DNA fragment is recovered from the vector for further analysis. To test whether genes identified by the two hybrid system are required for peroxisome biogenesis, the recovered genes will be used to create deletions in the gene and to determine whether the deletion strains are *per* mutants.

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