Fatty acid beta-oxidation in Saccharomices cerevisae: new insights with implications for human diseases

van Roermund, C.W.T.

Citation for published version (APA):
Chapter 8

Fatty acid β-oxidation in *Saccharomyces cerevisiae*: an overview.

Chapter 8: Fatty acid β-oxidation in *Saccharomyces cerevisiae* an overview

1 Fatty acid β–oxidation in *Saccharomyces cerevisiae*

1.1 Substrates for the β-oxidation system.

1.2 Enzymology of the peroxisomal β–oxidation.

1.3 The first step of the peroxisomal β–oxidation system.

1.4 The second and third step of the peroxisomal β–oxidation system.

1.5 The final step of the peroxisomal β–oxidation system.

1.6 Unsaturated fatty acid β–oxidation.

1.7 Induction of fatty acid β–oxidation

1.8 Peroxisomal membrane proteins (other than peroxins).

2 Metabolic transport across the peroxisomal membrane.

2.1 Transport of fatty acid.

2.1.1 Fatty acid transport across the plasma membrane

2.1.2 Fatty acid transport across the peroxisomal membrane

2.2 Shuttling of metabolites across the peroxisomal membrane

2.2.1 Reoxidation of intraperoxisomal NADH.

2.2.2 Reduction of intraperoxisomal NADP⁺.

2.2.3 Export of acetyl-CoA from the peroxisomal interior to the mitochondrial matrix during fatty acid oxidation.
Peroxisomal disorders are relative newcomers in the arena of inherited diseases in humans. In recent years many different peroxisomal disorders have been identified and great progress has been made with respect to the underlying enzymatic and genetic basis (Gould et al., 2000; Wanders et al., 1999). On the other hand many patients have been described in literature in which the underlying defect remains to be established. In many of these patients the defect appears to be in the peroxisomal β-oxidation system as concluded from the accumulation of very-long-chain fatty acids, pristanic acid and/or di- and trihydroxycholestanoic acid in different combinations.

Figure 1 Metabolic transport across the peroxisomal membrane during fatty acid oxidation in S. cerevisiae.
The recent identification of D-bifunctional protein deficiency (Suzuki et al., 1997; Van Grunsven et al., 1998, 1999a/b) and 2-methylacyl-CoA racemase deficiency (Ferdinandisse et al., 2000) has been of great importance in our endeavor to resolve the true underlying defect in all patients with a defect in peroxisomal β-oxidation of unknown etiology (Wanders et al., 2001b). In order to shed more light on the functional organization and basic principles of peroxisomal β-oxidation, we and others have used the yeast *S. cerevisiae* as model organism. The yeast *S. cerevisiae* is able to degrade both saturated and unsaturated fatty acids (Dommes et al., 1981). In contrast to higher eukaryotes, fatty acid β-oxidation in yeast is restricted to peroxisomes, which harbour the full enzymatic machinery to degrade fatty acids. Efficient oxidation of saturated and unsaturated fatty acids in peroxisomes does not only require the participation of a series of enzymes but also requires the active involvement of other gene products. Indeed, peroxisomes must be equipped with mechanisms to (1) reoxidize the NADH produced during fatty acid β-oxidation (2) to reduce the NADP⁺ produced in the 2,4-dienoyl-CoA reductase reaction (3) to take up fatty acids from the cytosol and (4) to export acetyl-CoA units out of the peroxisome to mitochondria for full oxidation to CO₂ and H₂O (Fig. 1) which is coupled to the synthesis of ATP.

1.1 Substrates for the β-oxidation system in *Saccharomyces cerevisiae*

**Saturated fatty acids**

*S. cerevisiae* can use a range of different saturated fatty acids including myristic acid (C14:0) and palmitic acid (C16:0) as sole carbon source. Some fatty acids, like lauric acid (C12:0) or octanoic acid (C8:0) are substrates for β-oxidation but are lethal when used as sole carbon source.

**Mono- and polyunsaturated fatty acids**

Peroxisomes are also able to oxidize unsaturated fatty acids including oleic acid (C18:1ω9), docosahexaenoic acid (C22:6ω3) and arachidonic acid (C20:4ω3). These substrates can be used as sole carbon source.

**Very long chain fatty acids**

Whereas long-chain fatty acids can be used as sole carbon source, *S. cerevisiae* can not grow on a very long chain fatty acid (VLCFA) like C26:0 as sole carbon source despite the fact that VLCFA like C24:0 and C26:0 can be oxidized in *S. cerevisiae* at a low rate (1% as compared with oleate) (Van Roermund et al., unpublished).

2-methylbranched chain fatty acids

*S. cerevisiae* can not β-oxidize pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) and other 2-methyl-branched chain fatty acids (Van Roermund et al., unpublished).

1.2 Enzymology of the peroxisomal β-oxidation system

The actual β-oxidation of fatty acids in *S. cerevisiae* proceeds via the same set of 4 reactions as in mitochondria and peroxisomes from higher eukaryotes. This implies that a fatty acid is first activated to a CoA ester and then undergoes dehydrogenation, hydration, isomerization, dehydrogenation again and thiol-lytic cleavage.

1.3 The first step of the peroxisomal β-oxidation system

The first step in the β-oxidation of acyl-CoA esters involves the introduction of a double bond between the α and β carbon atoms. In *S. cerevisiae* only a single acyl-CoA oxidase (Fox1p or Pox1p) (Dmochowska et al., 1990) has been identified which catalyzes the dehydrogenation of all acyl-CoAs. During this reaction, hydrogen peroxide is produced which is subsequently decomposed by catalase to produce H₂O and O₂. *FOX1* is the orthologue of the human acyl-CoA oxidase 1 gene, and its expression is strongly induced by fatty acids (Luo et al., 1996; Rottensteiner et al., 1996).
1.4 The second and third step of the peroxisomal \( \beta \)-oxidation system

The next two reactions, a hydration and a NAD\(^+\)-dependent dehydrogenation reaction, are catalyzed by a bi- or multi-functional protein with enoyl-CoA hydratase and 3-OH acylCoA dehydrogenase activity (Fox2p) (Hiltunen et al., 1992; Qin et al., 1999). Fox2p is the orthologue of the human D-bifunctional protein (D-BP), also known as MFP2, MFEII and D-PBE. Its expression is markedly induced by fatty acids. Both ScFox2p and HsMFP2p catalyze the formation of 3-ketoacyl-CoA esters from 2-enoylCoAs with D-3 hydroxyacylCoA as intermediate.

1.5 The final step of the peroxisomal \( \beta \)-oxidation system

The final reaction of the peroxisomal \( \beta \)-oxidation pathway is catalyzed by 3-ketoacyl-CoA thiolase (Fox3p or Pot1p)(Igual et al., 1991, Erdmann et al., 1994), which thiol-lytically cleaves 3-ketoacyl-CoA esters into a chain shortened acyl-CoA and acetyl-CoA. The expression of thiolase is also induced by fatty acids.

Until now, only one version of each enzyme has been described in *S. cerevisiae* and analysis of the *S. cerevisiae* Genome Database did not result in the identification of candidate isoenzymes (Van Roermund et al., unpublished).

1.6 Unsaturated fatty acid \( \beta \)-oxidation

\( \beta \)-oxidation of unsaturated fatty acids requires the participation of additional enzymes including \( \Delta^3, \Delta^2 \)-dienoyl-CoA isomerase (Dci1p), a 2,4-dienoyl-CoA reductase (Sps19p) (Gurvitz et al., 1997) and a \( \Delta^3, \Delta^2 \)-enoyl-CoA isomerase (Eci1p) (Geisbrecht et al., 1998).

Figure 2 depicts the degradation of unsaturated fatty acids and the involvement of the various enzymes. Three different routes can be distinguished. Route A is followed by fatty acids with unsaturations at even-numbered positions yielding 2,4-dienoyl-CoAs, which can only be further oxidized after conversion to 2-enoyl-CoAs by the sequential action of 2,4-dienoyl-CoA reductase and \( \Delta^3, \Delta^2 \)-enoyl-CoA isomerase (Fig. 2). In contrast, fatty acids with unsaturations at odd-numbered positions can follow two different routes. The first route depicted in Fig. 2 involves conversion of 2,5-dienoyl-CoAs into 2-enoyl-CoAs via the subsequent action of 1. Fox2p. 2. Fox3p and 3. \( \Delta^3, \Delta^2 \)-enoyl-CoA isomerase. The other NADPH-dependent pathway involves the sequential action of \( \Delta^3, \Delta^2 \)-enoyl-CoA isomerase, \( \Delta^3, \Delta^2, \Delta^4 \)-dienoyl-CoA isomerase, 2,4-dienoyl-CoA reductase, and, again, \( \Delta^3, \Delta^2 \)-enoyl-CoA isomerase to finally yield 2-enoyl-CoAs.

Efficient oxidation of both saturated and unsaturated fatty acids in peroxisomes requires the active involvement of other gene products. Indeed, the peroxisome must have mechanisms to (1) reoxidize the NADH produced in the Fox2p reaction; (2) to reduce the NADP\(^+\) produced in the 2,4-dienoyl-CoA reductase reaction; (3) to take up fatty acids from the cytosol and (4) to export the acetyl-CoA units out of the peroxisome via the carnitine pathway or the glyoxylate cycle to mitochondria for full oxidation to CO\(_2\) and H\(_2\)O (Fig 1). Table 1 lists the genes and corresponding gene products with a defined or presumed role in fatty acid \( \beta \)-oxidation.

1.7 Induction of the peroxisomal \( \beta \)-oxidation system

The induction of genes encoding peroxisomal proteins in yeast cells is mediated via the transcription factors Pip2p and Oaf1p, which are involved in the control of gene expression, especially of genes involved in the fatty acid \( \beta \)-oxidation. Remarkably, although proteins of the proteinimport machinery and many of the peroxisomal enzymes have been conserved from yeast to man, the mammalian (PPAR and RXR) and yeast transcription factors (Pip2p and Oaf1p) which control fatty acid oxidation have little in common.
Figure 2. Unsaturated fatty acid oxidation

The class of nuclear receptors to which PPARα belongs is unknown in yeast, and Zn₂Cys₆ trans-cription factors are typical of fungi (Chervitz et al., 1998). Transcription of genes encoding peroxisomal proteins in yeast can be induced in different ways. In methylotrophic yeasts like H. Polymorpha and P. pastoris, methanol is a potent inducer of peroxisomal enzymes, such as amine oxidase (Veenhuis and Harder, 1991). In S. cerevisiae, proliferation of peroxisomes and induction of the fatty acid β-oxidation machinery is required in order to grow on oleate. Promoters of genes coding for yeast proteins, which are involved in fatty acid oxidation, contain a positive cis-acting element that mediates the induction of these genes by fatty acids in the medium. This element is called an Oleate Response Element (ORE) (Einerhand et al., 1991) and is characterized as an imperfect inverted repeat containing conserved CGG triplets that are spaced by 14-19 nucleotides (CGG-N14/N19-CGG). Further studies demonstrated that Pip2p and Oaf1p interact with each other (Karpichev et al., 1998) and form a heterodimer that binds ORES (Rottensteiner et al., 1996) and is required for fatty acid-induced peroxisomal proliferation and regulates the expression of proteins required for fatty acid oxidation.

A high rate of synthesis of peroxisomal matrix proteins is not sufficient to induce proliferation of the peroxisomal compartment. This is illustrated by the overexpression of MDH3 under glucose conditions, which results only in the increased size of existing peroxisomes without inducing peroxisomal proliferation (Verleur et al., 1997a).
Therefore, other mechanisms are required to trigger peroxisomal proliferation. Previously, Pex11p had been implicated in the regulation of the number of peroxisomes (Erdmann and Blobel, 1995; Marshall et al., 1995; Sakai et al., 1995). Proteins with a low amino acid sequence similarity (20%) to S. cerevisiae Pex11p have been found in a wide variety of eukaryotes. It has been suggested that Pex11p is involved in a process leading to fission or vesiculation of pre-existing peroxisomes. An interesting observation supporting such a role of Pex11p was made by Passreiter et al., 1998.

Table 1 Foxins: genes encoding proteins involved in fatty acid \(\beta\)-oxidation in Saccharomyces cerevisiae.

<table>
<thead>
<tr>
<th>Foxin</th>
<th>Gene name</th>
<th>ORF</th>
<th>Localization</th>
<th>Pheno type</th>
<th>ORE-box</th>
<th>PTS</th>
<th>remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fox1p</td>
<td>FOX1</td>
<td>YGL205w</td>
<td>peroxisomal matrix</td>
<td>olete non utiliser</td>
<td>PIP2/OAF1 ADR1</td>
<td>not known</td>
<td>Acyl-CoA oxidase</td>
</tr>
<tr>
<td>Fox2p</td>
<td>FOX2</td>
<td>YKR009c</td>
<td>peroxisomal matrix</td>
<td>olete non utiliser</td>
<td>PIP2/OAF1 ADR1</td>
<td>PTS1 (SKL)</td>
<td>Bi- or multifunctional protein</td>
</tr>
<tr>
<td>Fox3p</td>
<td>FOX3</td>
<td>TIL160c</td>
<td>peroxisomal matrix</td>
<td>olete non utiliser</td>
<td>PIP2/OAF1 ADR1</td>
<td>PTS1 (SKL)</td>
<td>3-ketoacyl-CoA thiolase</td>
</tr>
<tr>
<td>Fox4p</td>
<td>SPS19</td>
<td>YNL202w</td>
<td>peroxisomal matrix</td>
<td>petroselinic non utiliser</td>
<td>PIP2/OAF1 ADR1</td>
<td>PTS1 (SKL)</td>
<td>2,4-dienoyl-CoA reductase</td>
</tr>
<tr>
<td>Fox5p</td>
<td>DCL1</td>
<td>YOR180c</td>
<td>peroxisomal matrix</td>
<td>viable on olete</td>
<td>PIP2/OAF1 ADR1</td>
<td>PTS1 (SKL)</td>
<td>(\Delta^{1,2})-dienoyl-CoA isomerase</td>
</tr>
<tr>
<td>Fox5p</td>
<td>ECL1</td>
<td>YLR284c</td>
<td>peroxisomal matrix</td>
<td>olete non utiliser</td>
<td>PIP2/OAF1 ADR1</td>
<td>PTS1 (SKL)</td>
<td>(\Delta^{1,2})-dienoyl-CoA isomerase</td>
</tr>
<tr>
<td>Fox7p</td>
<td>FAA1</td>
<td>YOR317w</td>
<td>cytosol</td>
<td>(\Delta\text{fai1/\Delta\text{fai4}}) olete non utiliser</td>
<td>-</td>
<td></td>
<td>acyl-CoA synthetase</td>
</tr>
<tr>
<td>Fox8p</td>
<td>FAA2</td>
<td>YER015w</td>
<td>peroxisomal matrix</td>
<td>MCFA non utiliser</td>
<td>PIP2/OAF1</td>
<td>PTS1 (SKL)</td>
<td>acyl-CoA synthetase</td>
</tr>
<tr>
<td>Fox9p</td>
<td>FAA4</td>
<td>YMR246w</td>
<td>cytosol</td>
<td>(\Delta\text{fai1/\Delta\text{fai4}}) olete non utiliser</td>
<td>-</td>
<td></td>
<td>acyl-CoA synthetase</td>
</tr>
<tr>
<td>Fox10p</td>
<td>FAT1</td>
<td>YBR041w</td>
<td>plasma and peroxisomal membrane</td>
<td>viable on olete</td>
<td>-</td>
<td>PTS1 (SKL)</td>
<td>very longchain acyl-CoA synthetase</td>
</tr>
<tr>
<td>Fox11p</td>
<td>FAT2</td>
<td>YBR222c</td>
<td>peroxisomal periphery</td>
<td>viable on olete</td>
<td>PIP2/OAF1 ADR1</td>
<td>PTS1 (SKL)</td>
<td>very longchain acyl-CoA synthetase</td>
</tr>
<tr>
<td>Fox12p</td>
<td>ACS1</td>
<td>YAL054c</td>
<td>peroxisomal matrix</td>
<td>also reported to be mitochondrial</td>
<td>ADR1</td>
<td>PTS1 (VKL)</td>
<td>acetyl-CoA synthetase</td>
</tr>
<tr>
<td>Fox13p</td>
<td>ACB1</td>
<td>YGR037c</td>
<td>cytosol</td>
<td>viable on olete</td>
<td>PIP2/OAF1 ADR1</td>
<td>-</td>
<td>acyl-CoA binding protein</td>
</tr>
<tr>
<td>Fox14p</td>
<td>TES1</td>
<td>YJR019c</td>
<td>peroxisomal matrix</td>
<td>viable on olete</td>
<td>PIP2/OAF1</td>
<td>PTS1 (AKF)</td>
<td>peroxisomal thioesterase</td>
</tr>
<tr>
<td>Fox15p</td>
<td>CAT2</td>
<td>YML042w</td>
<td>peroxisomal matrix</td>
<td>viable on olete</td>
<td>PIP2/OAF1</td>
<td>PTS1 (AKL)</td>
<td>carnitine acetyltransferase</td>
</tr>
<tr>
<td>Fox16p</td>
<td>CTA1</td>
<td>YDR256c</td>
<td>peroxisomal matrix</td>
<td>viable on olete</td>
<td>PIP2/OAF1 RTG1/RTG2</td>
<td>PTS1 (SKL)</td>
<td>catalase A</td>
</tr>
<tr>
<td>Fox17p</td>
<td>MDH2</td>
<td>YOL126c</td>
<td>cytosol</td>
<td>olete non utiliser</td>
<td>ADR1 RTG1/RTG2</td>
<td>-</td>
<td>malate dehydrogenase</td>
</tr>
<tr>
<td>Fox18p</td>
<td>MDH3</td>
<td>YDL078c</td>
<td>peroxisomal matrix</td>
<td>olete non utiliser</td>
<td>PIP2/OAF1 ADR1</td>
<td>PTS1 (SKL)</td>
<td>malate dehydrogenase</td>
</tr>
<tr>
<td>Fox19p</td>
<td>IDP2</td>
<td>YLR174w</td>
<td>cytosol</td>
<td>not viable</td>
<td>-</td>
<td></td>
<td>isocitrate dehydrogenase (NADP)</td>
</tr>
<tr>
<td>Fox20p</td>
<td>IDP3</td>
<td>YNL009w</td>
<td>peroxisomal matrix</td>
<td>petroselinic non utiliser</td>
<td>PIP2/OAF1</td>
<td>PTS1 (AKL)</td>
<td>isocitrate dehydrogenase (NADP)</td>
</tr>
<tr>
<td>Fox21p</td>
<td>CIT2</td>
<td>YCR005c</td>
<td>peroxisomal matrix</td>
<td>viable on olete</td>
<td>RTG1/RTG3</td>
<td>PTS1 (SKL)</td>
<td>citrate synthase</td>
</tr>
<tr>
<td>Fox22p</td>
<td>ACO2</td>
<td>cytosol</td>
<td>?</td>
<td></td>
<td>RTG1/RTG2</td>
<td>-</td>
<td>aconitase</td>
</tr>
<tr>
<td>Fox1</td>
<td>Gene name</td>
<td>ORF</td>
<td>Localisation</td>
<td>Phenotype</td>
<td>ORE-box</td>
<td>PTS</td>
<td>remarks</td>
</tr>
<tr>
<td>------</td>
<td>-----------</td>
<td>-----</td>
<td>--------------</td>
<td>-----------</td>
<td>---------</td>
<td>-----</td>
<td>---------</td>
</tr>
<tr>
<td>Fox23p</td>
<td>MLS1</td>
<td>YNL177w</td>
<td>peroxisomal matrix</td>
<td>viable on olate (partial)</td>
<td>RTG1</td>
<td>PTS1 (SKL)</td>
<td>Malate synthase</td>
</tr>
<tr>
<td>Fox24p</td>
<td>ICL1</td>
<td>YER065c</td>
<td>cytosolic</td>
<td>acetate non utiliser</td>
<td>RTG1/RTG2</td>
<td>-</td>
<td>isocitrate lyase</td>
</tr>
<tr>
<td>Fox25p</td>
<td>AAT2</td>
<td>YLR027c</td>
<td>cytosolic and peroxisomal</td>
<td>viable on olate</td>
<td>-</td>
<td>PTS1 (AKL)</td>
<td>Aspartate amino transferase</td>
</tr>
<tr>
<td>Fox26p</td>
<td>PXA1</td>
<td>YKL188c</td>
<td>PMP</td>
<td>olate non utiliser</td>
<td>PIP2/OAF1</td>
<td>-</td>
<td>ABC transporter</td>
</tr>
<tr>
<td>Fox27p</td>
<td>PXA2</td>
<td>YCR077c</td>
<td>PMP</td>
<td>olate non utiliser</td>
<td>PIP2/OAF1</td>
<td>-</td>
<td>ABC transporter</td>
</tr>
<tr>
<td>Fox28p</td>
<td>PMP35</td>
<td>YPR128c</td>
<td>PMP</td>
<td>viable on olate</td>
<td>PIP2/OAF1</td>
<td>-</td>
<td>FFA transport pathway</td>
</tr>
<tr>
<td>Fox29p</td>
<td>PMP31</td>
<td>PMP</td>
<td>PMP</td>
<td>-</td>
<td>PIP2/OAF1</td>
<td>-</td>
<td>Porin?</td>
</tr>
<tr>
<td>Fox30p</td>
<td>PEX11/ PMP27</td>
<td>YOL147c</td>
<td>peroxisomal periphery</td>
<td>olate non utiliser</td>
<td>PIP2/OAF1</td>
<td>-</td>
<td>FFA transport pathway</td>
</tr>
<tr>
<td>Fox31p</td>
<td>CAC</td>
<td>YOR100c</td>
<td>MIM</td>
<td>viable on olate</td>
<td>PIP2/OAF1</td>
<td>-</td>
<td>acylcarnitine/carnitine carrier</td>
</tr>
<tr>
<td>Fox32p</td>
<td>ACR1</td>
<td>YJR095w</td>
<td>MIM</td>
<td>viable on olate</td>
<td>PIP2/OAF1</td>
<td>-</td>
<td>fumarate/succinate carrier</td>
</tr>
<tr>
<td>Fox33p</td>
<td>AGP2</td>
<td>YBR132c</td>
<td>plasma membrane</td>
<td>viable on olate (rich)</td>
<td>PIP2/OAF1</td>
<td>-</td>
<td>carnitine carrier</td>
</tr>
</tbody>
</table>

Rat Pex11p has been shown to bind coatamer \textit{in vitro} by virtue of its cytoplasmically exposed carboxyl-terminal dilysine motif. Recruitment of coatamer by Pex11p has been proposed to initiate vesiculation of peroxisomes and thereby influence peroxisome proliferation (Passreiter \textit{et al.}, 1998). However, this dilysine motif is not conserved in other Pex11p homologs thereby raising doubt about the universality of the proposed mechanism for Pex11p-mediated peroxisomal fission. Recently we found that Pex11p is required for medium-chain fatty acid oxidation and primarily plays a metabolic role that affects peroxisomal fission (Chapter 7). Barnett \textit{et al.}, 2000 observed a highly significant amino acid sequence similarity (30% identity; 50% similarity) between amino acids 2-187 of Pex11p and the ligand binding domain (LBD) of the nuclear receptor PPAR\(\alpha\), which may suggest that Pex11p binds the same ligand. Its homology to the Ligand Binding Domain (LBD) of nuclear hormone receptors, especially PPAR\(\alpha\), is intriguing. Studies in human cells, as well as in the yeast \textit{Y. lipolytica}, \textit{C. boidinii}, and \textit{S. cerevisiae} support the hypothesis that \(\beta\)-oxidation is required for proper regulation of size and morphology of the peroxisomal compartment (Poll-Thé \textit{et al.}, 1988; Smith \textit{et al.}, 2000). In agreement with this postulate, we found defective peroxisome proliferation in \(\Delta\text{fox1}, \Delta\text{fox2}, \text{and}\ \Delta\text{fox3}\) cells (data not shown). Finally we found the occurrence of similar giant peroxisomes in the \(\Delta\text{yp128c}\) and \(\Delta\text{tes}\) (acyl-CoA thioesterase) mutant growing on olate. These observations suggest that YPR128cp, Tesp, as well as Pex11p and peroxisomal \(\beta\)-oxidation per se, are involved in the same metabolic pathway that is required for proper peroxisomal fission (Chapter 6 and 7).

1.8 Peroxisomal membrane proteins (other than peroxins)

Several peroxisomal membrane proteins (PMPs) other than peroxins, have been identified which are presumed to be involved in solute transport across the peroxisomal membrane (see Table 1). These include: PMP members of the mitochondrial carrier family (MCF).
The C. boidinii PMP47 gene encodes a 47 kDa integral protein which is homologous to mitochondrial solute carriers present in the mitochondrial membrane (Mc Cammon et al., 1990). The N- and C- termini of Pmp47p were shown to be exposed to the peroxisomal matrix (Mc Cammon et al., 1994). Thus, the proposed topology is inverted in comparison to mitochondrial carriers, because their termini extend into the mitochondrial intermembrane space (Kuan and Saier, 1993). Recently, putative human, mouse and S. cerevisiae homologues of Pmp47p, designated Pmp34p, have been identified. Similar to Pmp47p, six transmembrane domains were predicted in these proteins (Wylin et al., 1998). As discussed in chapter 7 this protein is probably involved in transmembrane ATP transport. In addition to the PMP47/PMP34 proteins, another peroxisomal member of the MCF family has been identified in the rabbit, namely a 53 kDa peroxisomal Ca2+-dependent solute carrier (Weber et al., 1997). No homologues were found in yeast of this protein.

Pxa1p and Pxa2p, two members of the ABC transporter family. Translocation of phospholipids across the plasma membrane of mammalian cells has been shown to be dependent on the action of the ABC transporters Mdr1p and Mdr3p (Smith et al., 1994; van Helvoort et al., 1996). These proteins have been postulated to function as flipases that bind an amphipathic lipid in the cytoplasmic leaflet of the plasma membrane and flip its polar group across the membrane to deliver the molecule to the exoplasmic leaflet (Higgins and Gottesman, 1992). In the genome of the yeast S. cerevisiae only two peroxisomal ABC transporters have been identified, namely Pxa1p and Pxa2p. These proteins are highly similar to PMP70p and ALDp.

Pmp20p and Pmp31p
Finally two other peroxisomal membrane proteins were identified in yeast, S. cerevisiae Pmp20p and H. polymorpha Pmp31p. The precise function of these PMPs is yet unknown.

The permeability properties of peroxisomes and the involvement of these different PMPs will be discussed in the next paragraph.

2 Transport of metabolites across the peroxisomal membrane

For most of the enzymatic pathways delineated so far, peroxisomes are dependent on efficient communication with the remainder of the cell. For continuation of β-oxidation, products have to be recycled and/or transported over the peroxisomal membrane. Originally the hypothesis was that peroxisomes would be freely permeable to low molecular weight compounds. This was concluded from the behavior of peroxisomes upon equilibrium density gradient centrifugation in sucrose and the finding that several enzymes such as D-amino acid oxidase failed to exhibit structurelinked latency (De Duve and Bauduin, 1966). Direct permeability measurements were done later using patch clamp analysis which provided support for the concept of peroxisomes being freely permeable organelles (Van Veldhoven et al., 1987). In 1993 Sulter et al., reported the presence of a porin in the peroxisomal membrane. Porins are known to be present in the outer membrane of mitochondria (Manella et al., 1992). Their molecular weight varies from 29-36 kDa. Although the porins exhibit little sequence conservation, their structural properties are very similar. Porins form a cylinder built from 12-19 amphiphatic β-sheets, which results in a barrel-like structure with an inner diameter of 1.8 to 2.5 nm. This allows the passage of molecules of up to 4-5 kDa.

The first protein which is supposed to be responsible for pore-forming activity in the rat is the 22kDa peroxisomal membrane protein (Pmp22p), which has a high
similarity with certain transmitter-gated ion channels, but has no similarity with porins, which makes the claim of a peroxisomal porin questionable. Recently, Tugal et al., 1999 sequenced and characterized PMP22 from Arabidopsis, which shares 55% similarity to two related mammalian peroxisomal membrane proteins, Pmp22p and Mpv17p. These proteins are postulated to be involved in redox reactions associated with the reoxidation of NADH, or in defense against potentially harmful reactive oxygen species such as hydrogen peroxide and superoxide radicals produced in matrix and membranes. These new results cast doubt on Pmp22p as a peroxisomal porin. In yeast no homolog of PMP22 has yet been identified, but interestingly Lee et al., 1999 and Farcasanu et al, 1999 characterized a Pmp20p (Ahp1p) in yeast, which is located in the periphery, as a new anti-oxidant with alkyl hydroperoxide defense properties in yeast.

The second protein which is supposed to be responsible for pore-forming activity is the H. polymorpha Pmp31p (Table 1), which is strictly Ca\(^{2+}\) regulated. In this respect the electrophysiological properties of Pmp31p clearly differed from that of several other porins, including the homologue of PMP31 in the mitochondrial outer membrane of H. polymorpha (Lemmens et al., 1989). Since the porin is predominantly present in the closed state at calcium concentrations below 10\(^{-6}\) M and the physiological calcium concentration in the cell is approximately 10\(^{-6}\) M, this suggests a possible regulation by calcium of the porin in vivo.

Thirdly, Corpas et al., 2000 identified a 36 kDa polypeptide of unknown function in the membrane fraction of cucumber seedling glyoxysomes. They presented evidence that this 36kDa protein revealed 72%-95% identities with sequences in mitochondrial porins of several different plant species. Earlier, Reumann et al., (1996) proposed that this peroxisomal porin-like channel activity contains a binding site for malate and oxaloacetate, which introduces the possibility that this porin-like activity is involved in the malate shuttle of leaf peroxisomes.

Although it remains to be resolved why isolated peroxisomes behave as freely permeable organelles, it is now well established that peroxisomes are closed compartments under *in vivo* conditions. Firstly we have found that the peroxisomal membrane is impermeable to NAD(H) and acetyl-CoA (Van Roermund et al., 1995) and NADP(H) (Van Roermund et al., 1998). These findings predict the existence of metabolite carriers in the peroxisomal membrane to shuttle metabolites from peroxisomes to cytoplasm and visa versa, especially for substrates and \(\beta\)-oxidation products during the \(\beta\)-oxidation of fatty acids. Secondly, recent studies by Dansen et al., 2000 have shown that peroxisomes are even impermeable to protons. Making use of a pH-sensitive probe targeted to peroxisomes via an oligopeptide equipped with a PTS1-signal, Dansen et al., demonstrated that peroxisomes have a basic pH (8.2+-0.3), which can be dissipated by uncoupler. The mechanism involved in the maintenance of this pH gradient, is unresolved.

### 2.1 Fatty acid transport

#### 2.1.1 Uptake and activation

Transport of unesterified fatty acids (FFA) has been proposed to proceed via simple diffusion mainly regulated by the rules of lipid physical chemistry. Recently, however a more complex process involving protein catalysis has been suggested. Hamilton et al., 1998 divided FFA transport in cell membranes into three essential steps: adsorption, transmembrane movement, and desorption. Because of their low solubility in water and high hydrophobicity, fatty acids bind rapidly and avidly to model
membranes; if albumin is a donor, FFA deresorbs rapidly to reach their equilibrium distribution. Deresorption of FFA from a phospholipid surface is slower than transmembrane movement and is dependent on the FFA chain length and unsaturation, but rapid for typical dietary FFA.

Each of these steps might be catalyzed by proteins in biological membranes, particularly if its rate is intrinsically slow. The physical properties of FFA in model systems predict that proteins are not essential for transport of FFA through membranes. Various factors can influence the rate of passive fatty acid movement across a membrane (1) a transmembrane pH gradient (Maloy et al., 1981) (2) the relative distribution of fatty acid binding sites on both sides of the membrane (Van Nieuwenhoven et al., 1996) (3) modification of free fatty acids to membrane impermeable derivatives (acyl-CoA esters) on the *trans*-side of the membrane (Klein et al., 1971; Schaffer et al., 1994); utilization of fatty acids for anabolic and catabolic processes, thereby creating a sink.

Fatty acids can cross the membrane either by virtue of their solubility or by being actively taken up by cells in a process mediated by proteins of the fatty acid transport (FATP) family. The protein encoded by the yeast *FAT1* gene has 54% overall similarity to human FATP and contains an AMP-binding motif common to such proteins as acyl-CoA synthetase. When the fatty acid synthase inhibitor cerulenin is added to the medium, yeast cells stop growing which can be rescued by supplementing different fatty acids. Cells carrying a disruption of the *FAT1* gene (*Δfat1*), however, have difficulty growing in the presence of cerulenin even in the presence of fatty acids. Incorporation of fatty acids into lipids was also impaired in the *Δfat1* cells. Thus in this initial report, Fat1p was proposed to function as a fatty acid transporter protein, as proposed for the murine FATP (Schaffer and Lodish, 1994; Faergeman et al., 1997). Recent observations, however, indicate that the impaired fatty acid uptake observed in the *Δfat1* cells is secondary to a defect in the metabolism of the fatty acid. Subcellular experiments suggest that Fat1p is associated with the endoplasmic reticulum and peroxisomal membrane, rather than the plasma membrane (Choi et al., 1999). Furthermore, heterologous expression of the *FAT1* gene indicates that Fat1p is a very-long-chain acyl-CoA synthetase (VLCS) (Choi et al., 1999). Fat1p appears to be involved in the maintenance of very-long-chain fatty acid homeostasis, only indirectly affecting utilization of exogenous fatty acids. It is interesting that the murine FATP has also recently been reported to be a VLCS (Coe et al., 1999).

Five additional genes (*FAA1-4* and *FAT2*) encoding proteins with homologies to acyl-CoA synthetases have been described in *S. cerevisiae* (Watkins et al., 1998; Blobel et al., 1996).

The *FAA1* and *FAA4* genes encode acyl-CoA synthetases (Table 1) required for activation of imported exogenous fatty acids (Duronio et al., 1992; Johnson et al., 1994; Knoll et al., 1994). Faa1p and Faa4p account for 99% of total 14:0 and 16:0 activation activity in *S. cerevisiae*, and when endogenous fatty acid synthesis is blocked, at least one is required for rescue on medium containing exogenous fatty acids (Knoll et al., 1994). Cells carrying disruptions in both genes, *Δfaa1/4aa4*, appear to have normal initial rates of free fatty acid import (Knoll et al., 1994), but bulk accumulation in cell lipids is negligible (Choi et al., 1999). Thus, it seems that these cells are defective in the activation, but not the transport, of the fatty acids. However, we found that the double knock-out (*Δfaa1/4aa4*) fails to grow on oleate as sole carbon source (Van Roermund, unpublished),
which suggests the involvement in fatty acid metabolism.

Disruption of the FAAT3 and FAAT2 genes has no affect on the ability of cells to use exogenously supplied fatty acids, which may suggest that the acyl-CoA synthetases encoded by these genes can access only fatty acids synthesized within the cell (Johnsson et al., 1994). Faat2p has been localized to the matrix side of the peroxisomal membrane (Hettema et al., 1996) and accounts for the residual VLCS activity present in cells lacking Fatlp (Choi et al., 1999).

The FAT2 gene product (Fat2p, previously named Psc60p) is located in the peroxisomal matrix but is not necessary for growth on oleic acid (Blobel et al., 1996). The cellular role of Faat3p and Fat2p remain uncertain.

In the cytoplasm acyl-CoA esters and free fatty acids are bound to binding proteins (ACBP and FABPs). Therefore the actual soluble acyl-CoA and fatty acid concentration is very low. Besides protecting the cellular membranes from fatty acids, FABPs and ACBPs have been postulated to play a role in the delivery of fatty acids and acyl-CoA esters to various compartments and fatty acid consuming systems (Glatz and van der Vusse, 1996; Gossiet et al., 1996; Faergeman and Knudsen, 1997).

2.1.2 Fatty acid transport across the peroxisomal membrane

The substrates for β-oxidation enter the peroxisome via two different pathways (Fig 1)(Hettema et al., 1996). First of all, free fatty acids like MCFAs, enter peroxisomes and are subsequently activated via the peroxisomal acylCoA synthetase, Faat2p. Fatlp is also required for β-oxidation of fatty acids that enter peroxisomes as free fatty acids. As discussed before, Fatlp shows extensive amino acid sequence similarity to the ligand-binding domain (LBD) of the nuclear hormone receptor (Barnett et al., 2000), suggesting that Fatlp might contain a binding site for fatty acids which would be in line with a role in fatty acid transport across the peroxisomal membrane.

The second entry path of fatty acids into the peroxisomes is by the activation of LCFAs outside peroxisomes and subsequent uptake via Pxa1p/Pxa2p (Hettema et al., 1996; Shani et al, 1995). Pxa1p and Pxa2p are peroxisomal membrane proteins that comprise the two halves of an ABC transporter required for import of activated long-chain fatty acids into peroxisomes. Pxa1p/Pxa2p might function as an acyl-CoA flippase in the peroxisomal membrane (Verleur et al., 1997). Membrane inserted acyl-CoA esters could then diffuse in the plane of the bilayer until they bind Pxa1p/Pxa2p, which flip the polar CoA group from the cytoplasmic leaflet to the luminal leaflet of the peroxisomal membrane. Subsequently β-oxidation of the acyl-CoA esters inside peroxisomes will allow a net flux of long-chain acyl-CoA esters into peroxisomes.

2.2 Shuttling of metabolites across the peroxisomal membrane

2.2.1 Reoxidation of intraperoxisomal NADH

As described in detail in Chapter 2 we have obtained strong evidence for a major role of peroxisomal Malate dehydrogenase (Mdhp3p) in intraperoxisomal NAD⁺ regeneration. Indeed deletion of the MDH3 gene, encoding peroxisomal malate dehydrogenase, blocks fatty acid oxidation in intact cells and leads to the accumulation of 3-hydroxyacyl-CoA esters. These results were taken to indicate that Δmdh3 cells are blocked at the NAD⁺ dependent dehydrogenation step. Furthermore, these results imply that NAD⁺ and NADH are not able to diffuse across the peroxisomal membrane. The Mdh3p-dependent regeneration of
Fatty acid β-oxidation in Saccharomyces cerevisiae: An overview

NAD⁺ depends on the presence of oxaloacetate inside peroxisomes. It has been hypothesized that the reduction equivalents are shuttled via an aspartate/malate shuttle, similar, to the situation in mitochondria (Fig. 3). The additional enzyme activity required for this shuttle, i.e. aspartate aminotransferase, was found to be localized inside peroxisomes, at least partially, under olate conditions (Verleur et al., 1997). However, the malate/aspartate shuttle is not required for fatty acid β-oxidation since disruption of the corresponding gene (AAT2) did not affect fatty acid β-oxidation (Verleur et al., 1997). The finding that Δmdh3 cells are not impaired in growth on acetate suggests that Mdh3p does not participate in the glyoxylate cycle. Indications that the kinetic parameters of glyoxysomal malate dehydrogenase are unfavorable to its participation in the glyoxylate cycle of plants glyoxysomes were earlier reported by Mettler and Beevers, 1980. The consequence of these findings is that malate produced by the glyoxylate cycle is transported out of the peroxisome followed by retro conversion to oxaloacetate in the cytosol (via Mdh2p) or mitochondria (via Mdh1p) (Van Roermund and Wanders, 2001).

2.2.2 Reduction of intraperoxisomal NADP⁺

β-Oxidation of some unsaturated fatty acids requires the participation of the enzyme 2,4-dienoyl-CoA reductase, which converts 2,4-dienoyl-CoA into Δ²-enoyl-CoA with the concomitant production of NADP⁺ from NADPH. As described in detail in Chapter 3, we have found that a peroxisomal NADP-linked isocitrate dehydrogenase activity plays a key role in the provision of NADPH required in the dienoyl-CoA reductase reaction. Disruption of the IDP3 gene was found to block the β-oxidation of unsaturated fatty acids containing an even-numbered double bond (Van Roermund, 1998) (Chapter 3). Under these conditions there was accumulation of 2,4-dienoyl-CoAs indicating that the metabolism of these fatty acids was blocked at the level of the dienoyl-CoA reductase step. β-oxidation of unsaturated fatty acids with an odd-numbered double bond (like oleate), however was unaffected, implying that these unsaturated fatty acids can be degraded independently of a NADPH-dependent reduction of 5-enoyl-CoA, supporting a role for the isomerase- or di-isomerase dependent pathway in vivo.

2.2.3 Export of acetyl-CoA from the peroxisomal interior to the mitochondrial matrix

As described in chapter 2 the peroxisomal citrate synthase activity (Cit2p) can be eliminated without impairment of growth on acetate and olate. This is rather surprising considering the essential role of the glyoxylate cycle discussed before. It has been observed and argued before that citrate produced in mitochondria under certain conditions can reach the peroxisomes for further metabolism. Here we suggest that the assimilation of acetyl-CoA can take place in a somewhat modified form which bypasses the need of Cit2p. The lack of an olate-non-utilizer (OUN) phenotype in the Δcit2 cells could be explained by the presence of an alternative pathway for transport of the produced acetyl-CoA units during β-oxidation, formed by the carnitine acetyltransferase protein (Cat2p). Disruption of both the CIT2 and CAT2 genes blocked the β-oxidation of olate in S. cerevisiae. These results led us to postulate two different routes for transport of β-oxidation
products from peroxisomes to mitochondria (Van Roermund et al., 1995); one via the glyoxylate cycle, and another one via the carnitine transport pathway (Fig. 5). Based on the findings, we developed a selection screen for the isolation of mutants that are specifically disturbed in the carnitine-dependent transport of acetyl-units from peroxisomes to the mitochondria. We report in chapter 4 the isolation and characterization of three groups of mutants which are all affected in proteins involved in the carnitine-dependent acetyl-CoA transport (CDAT) pathway from peroxisomes to the mitochondria.

2.2.4 ATP transport across the peroxisomal membrane

In chapter 7 we describe the role of YPR128cp, the orthologue of human PMP34, in fatty acid metabolism and peroxisomal proliferation in *Saccharomyces cerevisiae.*
YPR128cp belongs to the mitochondrial carrier family of solute transporters (MCF) and is localized in the peroxisomal membrane. Disruption of the YPR128c gene results in impaired growth of the yeast with the medium fatty acid (MCFA) laurate as a single carbon source, whereas normal growth was observed with the long-chain fatty acid (LCFA) oleate. These results imply that a transport step specific for MCFA β-oxidation is impaired in ypr128cΔ cells. Since MCFA β-oxidation in peroxisomes requires both ATP and CoASH for activation of the MCFAs into their corresponding CoA-esters, we studied whether YPR128cp is an ATP carrier (Fig. 1). For this purpose we have used firefly luciferase targeted to peroxisomes to measure ATP consumption inside peroxisomes. We show that peroxisomal luciferase activity was strongly reduced in intact ypr128cΔ mutant cells when compared to wild-type cells but comparable in lysates of both cell strains. We conclude that YPR128cp most likely mediates the transport of ATP across the peroxisomal membrane. Recently, direct evidence for the role of Ypr128cp as ATP-transporter was described by Palmieri et al., 2001. Who proposed that Ypr128cp catalyses a 1:1 exchange between ATP and AMP. 

In summary, much has been learned in recent years about the fatty acid oxidation system in yeast including the transport of fatty acids and other metabolites required for β-oxidation across the peroxisomal membrane. Much remains to be learned, however, especially about the exact way of transport of various metabolites across the membrane.