Metabolite transport across the peroxisomal membrane
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Introduction
Chapter 1

Introduction

Peroxisomes were first described by Rhodin in 1954 [1], who observed small apparently distinct organelles in mouse kidney cells which he called ‘microbodies’. In 1966, de Duve and Baudhuin [2] discovered the presence of catalase and H$_2$O$_2$-generating oxidases in these organelles, and introduced the name ‘peroxisome’. A similar organelle was discovered shortly thereafter by Breidenbach and Beevers [3] in the seeds of germinating plants, which was named ‘glyoxysome’ since it contained several of the enzymes involved in the glyoxylate cycle. It was subsequently shown that these organelles also contain many of the enzymes characteristically found in mammalian peroxisomes such as catalase, β-oxidation enzymes and H$_2$O$_2$ producing oxidases, which indicated that the organelles are closely related to the peroxisomes discovered earlier. Another related organelle was found by Oppendoes and Borst in 1977 in Trypanosoma parasites, which they named ‘glycosomes’ [4]. Although glycosomes do not contain H$_2$O$_2$ producing oxidases, they do contain β-oxidation enzymes and catalase, are similar in morphological appearance and have comparable sedimentation properties as mammalian peroxisomes [5].

The significance of peroxisomes in metabolism remained unclear for a long time. Even though Goldfischer et al [6] observed that patients suffering from Zellweger syndrome (ZS) lack discernible peroxisomes already in 1973, insight in the significance of this organelle only came a decade later when Brown et al [7] reported elevated levels of very-long-chain fatty acids in plasma from these patients, and Heymans et al [8] found a deficiency of plasmalogens in erythrocytes and tissues from ZS patients.

Currently, it is known that mammalian peroxisomes are involved in fatty acid α-oxidation and β-oxidation, the biosynthesis of etherphospholipids and bile acids and in the degradation of purines, polyamines, L-pipeolic acid and D-amino acids [9]. Metabolic functions of peroxisomes in other organisms include methanol degradation (yeast), glyoxylate cycle (plants, yeasts), penicillin biosynthesis (fungi), and karyogamy [10-12]. Although it was previously thought that peroxisomes also play a role in cholesterol and dolichol synthesis, it has recently been shown that this is not the case [13-18].

Defects in peroxisomal functioning are the cause of a number of severe human disorders, illustrating the essential role peroxisomes play in human metabolism. This is not further discussed in this thesis, but a number of recent reviews are available [19-22].

1.1 Peroxisomal biogenesis and protein import

The peroxisome number in a cell can vary widely, depending on cell type and conditions. Peroxisomes can be generated or degraded by the cell as required. Several lines of evidence indicate that peroxisomes can be generated by means of two processes: (i) fission of existing peroxisomes and (ii) budding from the endoplasmatic reticulum. A detailed description of our knowledge of these processes is beyond the scope of this thesis, but several reviews have been written on this topic [23-27]. The newly formed peroxisomes can acquire membrane and matrix proteins as required for cellular metabolism. Peroxisomes can eventually be degraded by a specific form of autophagy called pexophagy [28, 29]. An increasing number of genes are being discovered that appear to play a role in peroxisomal biogenesis, maintenance or degradation (PEX genes). To date, 32 of these genes have been identified in various organisms. However, with few exceptions very little is known about the precise function of the individual PEX genes and the proteins encoded by these genes, called ‘peroxins’. Most of the PEX genes have been identified in genetic screens designed to identify genes required for fatty acid β-oxidation in yeast. However, it seems that many newly discovered genes have been categorized as PEX genes simply because a gene disruption mutant has abnormal peroxisome morphology, and not
because it was found to have a proven function in organelle biogenesis. Therefore, it cannot be ruled out that the primary defect in some of the PEX gene disruption mutants is actually metabolic in nature and that abnormal peroxisome morphology is a consequence of the metabolic defect.

1.1.1 Peroxisomal matrix proteins
Matrix proteins are recognized by the peroxisomal import machinery by either of two targeting sequences: PTS1 or PTS2. PTS1 targeted proteins associate with the cytosolic receptor Pex5p. Presumably, the complex is imported into the peroxisome in its entirety, after which Pex5p is recycled to the cytosol while the PTS1 protein remains in the peroxisomal matrix [30]. The PTS1 signal is unusual in its simplicity and consists of a tripeptide sequence at the C-terminus of a protein, typically Serine-Lysine-Leucine (SKL), but variations thereof are accepted within limits. Several studies have been performed to arrive at a consensus sequence, but it is likely that the acceptable variations of the PTS1 signal are species specific, depending for instance on differences in Pex5p [31]. Using the import of malate dehydrogenase into peroxisomes of Saccharomyces cerevisiae as a model, the consensus sequence [AECPSHK][KHYR][MFIL] for the C-terminal tripeptide has been proposed [32]. However, the tertiary structure of the protein and specific residues in the region adjacent to the PTS1 tripeptide may alter the effectiveness of this signal Maynard et al [33] studied the binding affinity of human Pex5p for PTS1 containing peptides and found that binding is greatly favoured by a lysine at the –4 position, and leucine at the –5 position. In agreement, Mizuno et al [34] found that the lysine at the –4 position is required for the effective targeting of rat liver serine:pyruvate aminotransferase into peroxisomes. Amery et al [35] reported that the lysine at –4 is critical for the peroxisomal targeting of human 2-methyl-acyl-CoA racemase. The S. cerevisiae protein Amd2p, encoding a putative fatty acid amidase, contains two internal PTS2 consensus sequences as well as a classical PTS1 but is nevertheless localized to the cytosol (Visser et al, unpublished observations), suggesting that the tertiary structure prevents effective association of these targeting signals with the respective cytosolic receptors. Furthermore, it has been shown that for some proteins disruption of the PTS1 signal does not necessarily block targeting to the peroxisome. Examples of such proteins are alcohol oxidase in the yeast Hansenula polymorpha [36] and isocitrate lyase in castor bean [37]. However, even in the absence of a PTS1, these proteins still depend on Pex5p for peroxisomal targeting, suggesting that the association of these proteins with Pex5p is mediated by another domain. Indeed, native proteins without a PTS1 or PTS2 can be imported through another interaction with Pex5p or Pex7p. An example is acyl-CoA oxidase (Pox1p) of S. cerevisiae which does not have a canonical PTS1 or PTS2, but is nevertheless imported into peroxisomes. Klein et al [38] showed that an interaction with Pex5p is mediated by an internal domain located in the amino-terminal part of Pox1p. Baumgart et al [39] demonstrated that two specific isoforms of lactate dehydrogenase (A, and A,B) are present in the matrix of rat liver peroxisomes, even though they lack a PTS1 or PTS2. However, they did not report if lactate dehydrogenase is targeted via the Pex5p or Pex7p pathway. Furthermore, in chapter 2 of this thesis, the peroxisomal localization of glutathione reductase (Ghr1p) in S. cerevisiae is discussed, a protein which also does not possess a PTS1 or PTS2 sequence. Lastly, Lee et al [40] found that the S. cerevisiae glyoxylate cycle enzyme Cit2p is targeted to peroxisomes via a Pex5p and Pex7p-independent pathway. The targeting of this protein appears to be guided by a targeting signal near the amino terminus.

PTS2 targeted proteins bind the cytosolic receptor Pex7p. In mammals and Arabidopsis thaliana, Pex7p subsequently associates with a specific isoform of Pex5p [41-43]. Curiously, it appears that in S. cerevisiae, Pex7p complexes with Pex18p and Pex21p, and not with Pex5p [44]. Pex18p and Pex21p have not been identified in humans, and may be absent altogether. The PTS2 signal consists
of a nonapeptide located near the amino terminus of the protein. The allowable variations of the PTS2 signal have not been characterized as extensively as in the case of the PTS1 signal. However, very recently the consensus sequence [RK][LVQ][XX][LVHQ][LSGA][X][HQ][LA] was reported to describe essentially all functional variants known of the PTS2 signal [45]. Previously, the more general consensus sequence [RK][LV][X][HQ][LA] has been reported by Swinkel et al [46]. All currently known PTS2 targeted proteins are processed after delivery into the peroxosomal matrix. An amino-terminal presequence is removed, which is presumably performed by a specific protease which has not been identified yet.

Usage of the PTS2 signal for peroxosomal targeting seems surprisingly rare. The vast majority of peroxosomal matrix proteins is targeted by means of a PTS1 signal. Assuming that the majority of peroxosomal matrix proteins is currently known, the evolutionary advantage of preserving the PTS2 pathway for so few proteins is unclear, particularly considering the observation that in Caenorhabditis elegans, the Pex7p/PTS2 pathway is absent [47], and that all peroxosomal proteins in this organism seem to have acquired a PTS1 signal in the course of evolution.

1.1.2 Piggy-backing

Remarkably, matrix proteins can be imported into the peroxisome in a fully folded state, and even as large multimeric complexes. Walton et al [48] demonstrated that fully folded proteins, like IgG molecules, and even colloidal gold particles conjugated to proteins containing a PTS1 signal can be imported into peroxisomes. It may therefore be hypothesized that some proteins that do not contain a PTS1 or PTS2 themselves are nevertheless imported into the peroxisome through association with another protein that does have a peroxisomal targeting signal. This phenomenon is known as ‘piggy-backing’. Although no natural examples of this import mechanism are known, the possibility has been convincingly demonstrated for both PTS1 and PTS2 proteins. For instance, the peroxisomal targeting of S. cerevisiae Δ1,Δ2-enoyl-CoA isomerase (Eci1p) proceeds normally even if its PTS1 signal is disrupted [49], which was shown by Yang et al [50] to be mediated by association with Δ3,5-Δ2,4-dienoyl-CoA isomerase, a PTS1-targeted protein. Native thiolase in Nicotiana tabaccum L. exists as a dimeric complex, and is targeted by means of a PTS2 signal Fynn et al [51] demonstrated that epitope-tagged thiolase of which the PTS2 signal has been disrupted is co-imported into peroxisomes as a dimer with its native PTS2-bearing partner. Mutations that disturb dimerization also prevent peroxisomal import of the epitope-tagged protein. Similarly, catalase forms octamers which are imported into peroxisomes of the yeast Candida boidinii even if not all members of the complex posses a PTS1 [52]. Lastly, Nilsen et al [53] exploited the phenomenon of piggy-backing to demonstrate a physical interaction between two proteins by affixing a PTS1 to either one, which led to peroxisomal import of the protein complex.

Considering the possibility of piggy-backing, it may be that some of the alternative topogenic signals that have been investigated for peroxisomal matrix proteins lacking a PTS1 or PTS2 actually mediate an interaction with a partner that is in turn targeted by a PTS1 or PTS2 signal. Very little is known about the mechanism of the translocation of proteins across the peroxisomal membrane. The presence of a large opening in the peroxisomal membrane to allow protein translocation seems difficult to reconcile with the presence of ion-gradients and other apparent permeability properties of the peroxisomal membrane in-vivo. However, several lines of evidence suggest that peroxisomes do not import matrix proteins continuously but only for a relatively short period after their formation. Firstly, Waterham et al [54] demonstrated that in the yeast Candida boidinii a heterogeneous population of peroxisomes is formed when cells that have been grown on oleate containing medium are transferred to a medium containing methanol. It was observed that the organelles that were originally present and contained the β-oxidation enzymes required for oleate
metabolism did not acquire the enzymes required for methanol utilization. Instead, a new population of peroxisomes was formed that contained the newly synthesized peroxisomal proteins. Secondly, pulse chase experiments in rats have shown that newly synthesized acyl-CoA oxidase is first imported into a subpopulation of peroxisomes of intermediate density, and only later appears in high-density mature peroxisomes [55]. Therefore, the possibility exists that protein import, perhaps through a large opening in the membrane, does not take place in metabolically active peroxisomes. This would be consistent with the observed metabolite permeability of the peroxisomal membrane in-vivo.

Alternatively, the import of peroxisomal matrix proteins may proceed by a process that leaves the peroxisomal membrane barrier uncompromised. A mechanism comparable to endocytosis can be envisioned, i.e. invagination of the peroxisomal membrane, separation of a vesicle, and subsequent release of its contents. Future research on the mechanism of protein translocation should shed more light on how the integrity of the peroxisomal membrane towards metabolites can be reconciled with the translocation of large protein complexes.

1.1.3 Peroxisomal membrane proteins

The targeting process for peroxisomal membrane proteins (PMPs) is poorly understood. Current data seems to indicate that some PMPs are sorted via the endoplasmatic reticulum, whereas others are transcribed in the cytosol and subsequently inserted into the peroxisomal membrane. For instance, cottonseed (Gossypium hirsutum) ascorbate peroxidase appears to be sorted to peroxisomes through a specific subdomain of the endoplasmatic reticulum [56], whereas A. thaliana PMP22 is transcribed in the cytosol and posttranslationally inserted into the peroxisomal membrane [57]. Several studies have shown that some of the peroxisomal membrane proteins are present in peroxisomes already in a very early stage of biogenesis, prior to the import of matrix proteins [58]. This is not surprising, considering that several of the integral membrane PEX proteins are most likely part of the import machinery for matrix proteins.

Studies on the targeting of PMP22, human PMP34, C. bohadinii PMP47, human PMP70, PEX3, PEX11β and PEX22 have yielded evidence for the presence of targeting signals contained either in internal protein regions or near the amino terminus of the PMPs [59-66]. Other studies suggested the presence of multiple targeting signals in human and rat PMP22, S. cerevisiae Ant1p, human PMP34 and human PEX13 [67-70].

Several of the investigated PMPs contain a well conserved short stretch of positively charged residues that may be important for targeting [57, 66]. Disruption of conserved residues in these sites disturbs proper targeting of PMP34 [66] and PEX13 [70].

It has been suggested that Pex19p plays an important role in the sorting of PMPs to the peroxisomal membrane, for several reasons. Firstly, Pex19p interacts with a broad range of peroxisomal membrane proteins [64]. Gould and co-workers reported that the interaction domain of a number of PMPs coincided with a region that was previously found to be required for targeting [68, 71]. Recently, Rottensteiner et al [70] studied these sequence elements in more detail and found that disruption of conserved residues in these sites resulted in mislocalization of the respective proteins. In contrast, Fransen et al [72] reported that Pex19p-PMP interactions take place in regions distinct from those involved in targeting. In several of the investigated proteins, a domain involved in targeting was found to contain a evolutionarily conserved short cluster of positively charged amino acids which contributed significantly to the interaction with Pex19p [69-71]. Intriguingly, intentional mislocalization of Pex19p to the nucleus by the introduction of a nuclear targeting signal results in nuclear accumulation of newly synthesized PMPs [64]. Lastly, Pex19p is bimodally distributed between the cytoplasm and peroxisome, similar to what is observed for Pex5p and Pex7p, suggesting that it could be involved in a comparable shuttle-type mechanism for the sorting of PMPs.
Alternatively, it has been suggested that Pex19p may function as a chaperone for PMPs, and assist in maintaining the solubility of cytosolically transcribed PMPs and their eventual insertion into the peroxisomal membrane [71]. Although some progress was made in the aforementioned study by Rottensteiner et al [70] towards elucidation of the sequence elements involved in the targeting of membrane proteins, their consensus sequence appears not to be sufficiently specific to identify peroxisomal membrane proteins by sequence information alone. Unfortunately, the unavailability of a well-defined targeting sequence for membrane proteins greatly hinders in-silico searches for unknown peroxisomal membrane proteins, and hence of proteins potentially involved in metabolite transport.

1.2 Peroxisomal metabolism
Peroxisomes can participate in multiple metabolic pathways, depending on the cellular requirements. The expression of peroxisomal proteins may vary considerably, and is optimized to match the metabolic need. For instance, in S. cerevisiae, the presence of an oleate-responsive-element (ORE box) in the promoter of some peroxisomal proteins ensures a high expression level of these genes when fatty acids are provided as the sole carbon source. However, when glucose is provided, these genes are strongly repressed, and the encoded proteins are hardly detectable. A similar mechanism operates to induce the expression of alcohol oxidase in methylotrophic yeasts. Expression of the PEX genes responsible for peroxisomal biogenesis is affected surprisingly little by the environmental conditions [73].

Depending on the species, cell type and environmental conditions, peroxisomes may be involved in fatty acid α- and β-oxidation, biosynthesis of ether-phospholipids, and in the degradation of purines, polyamines and D-amino acids [9]. In the following paragraphs, the implied consequences for metabolite transport of the peroxisomal localization of these metabolic pathways will be discussed.

1.3 Fatty acid β-oxidation
β-oxidation is a cyclic process by which fatty acids are degraded from the carboxyl- end. Each cycle of β-oxidation shortens the fatty acid carbon chain by 2 carbon atoms, releasing an acetyl-CoA unit (or a propionyl-CoA unit if a 2-methyl group is present). One cycle of β-oxidation in peroxisomes consists of four reactions (figure 1), catalysed by three proteins, at least in S. cerevisiae. The β-oxidation of unsaturated fatty acids requires auxiliary enzymes for the elimination of double bonds [74, 75]. Degradation of unsaturated fatty acids with odd-numbered double bonds requires a Δ^2,Δ^4-enoxy-CoA isomerase. The degradation of fatty acids with even-numbered double bonds, requires 2,4-dienoyl-CoA reductase, in addition to the aforementioned isomerase. Two alternative pathways for the breakdown of fatty acids with a double bond at an odd-numbered position have been identified. Both rely on the activity of Δ^1,Δ^3-dienoyl-CoA isomerase, which is bimodally distributed between mitochondria and peroxisomes. In S. cerevisiae, the peroxisome is the exclusive site of fatty acid β-oxidation, while in mammals a β-oxidation system exists in both mitochondria and peroxisomes. Among other things, this property of S. cerevisiae renders it a very useful model organism to study peroxisomal β-oxidation. In mammals, the peroxisomal β-oxidation system differs from the mitochondrial system in several aspects.

Firstly, the initial step in peroxisomal β-oxidation is catalysed by H$_2$O$_2$ producing acyl-CoA oxidases, whereas the corresponding reaction in mitochondria is catalysed by FAD-linked acyl-CoA dehydrogenases. Consequently, the energy yield of peroxisomal β-oxidation in terms of oxidative phosphorylation is lower, because the energy contained in H$_2$O$_2$ is released as heat when it is degraded by catalase. In mitochondria, electrons are transferred to the electron-transfer-flavoprotein (ETF) and thus eventually to the respiratory chain.
Figure 1: Schematic representation of the peroxisomal β-oxidation. Each cycle shortens the fatty acid by 2 carbon atoms. Acetyl-CoA units are formed, which are transferred to the mitochondrion where they enter the citric acid cycle.
Secondly, the uptake of fatty acids into the peroxisomal matrix proceeds differently. Transport of fatty acids into the mitochondrion occurs by conjugation to carnitine (by carnitine palmitoyl transferase, CPT1), subsequent translocation of the carnitine ester across the mitochondrial membrane (by the carnitine:acylcarbamine carrier, CACT), and finally reformation of a CoA ester inside the mitochondrial matrix (by CPT2). It is currently believed that carnitine is not involved in the import of fatty acids in the peroxisomes. Evidence suggests that fatty acids can enter the peroxisome either as free acid, or directly as a CoA ester. This issue is further elaborated upon in section 1.6.5

Thirdly, the substrate specificity of the peroxisomal β-oxidation system differs from the mitochondrial system. Some fatty acids are degraded exclusively in mitochondria, some are a substrate of both peroxisomal and mitochondrial β-oxidation while other substrates are exclusively oxidized in peroxisomes.

Short chain fatty acids cannot be degraded in human peroxisomes. Consequently, as fatty acids are being shortened through peroxisomal β-oxidation, they become an increasingly worse substrate for the peroxisomal system. Eventually, the remaining fatty acid is exported from the peroxisome and transferred to the mitochondria to be degraded to completion. Evidence suggests that export from the peroxisome takes place in the form of a carnitine ester, which can then be directly imported into mitochondria. This topic is discussed in more detail in section 1.6.7.

The biosynthesis of the highly unsaturated fatty acids C22:5n-6 and C22:6n-3 (docosaheaxanoic acid, DHA) from dietary linoleate and linolenate (C18:3n-3) respectively proceeds by a series of anabolic and catabolic reactions requiring the involvement of both the endoplasmatic reticulum and peroxisomes [76]. Elongation and desaturation reactions in the endoplasmatic reticulum produce C24:5n-6 and C24:6n-3, which are subsequently β-oxidized in peroxisomes to produce C22:5n-6 and C22:6n-3. How the transfer of intermediates from ER to peroxisomes and the export from the peroxisome is accomplished is unknown. Interestingly, DHA formation from C20:5n-3 was reported to be normal in XALD patient fibroblasts, but impaired in Zellweger fibroblasts suggesting that ALDP is not involved in the transport of these compounds into or out of the peroxisome [77, 78].

In addition to fatty acids, the peroxisomal β-oxidation system also accepts a number of other substrates.

The bile acid precursors di- and trihydroxycholestanolic acid (DHCA and THCA) are subjected to one cycle of peroxisomal β-oxidation to yield chenodeoxycholoyl-CoA and cholooyl-CoA respectively. These are subsequently converted into the corresponding glycine- or taurine-esters by the peroxisomal enzyme bile acid CoA:amino acid N-acyltransferase (BAT) [79]. The peroxisomal localization of this enzyme implies an import mechanism for glycine and taurine, which has not been identified to date. Glycine can potentially be produced from alanine and glyoxylate by the action of the peroxisomal enzyme alanine:glyoxylate aminotransferase. No studies are available on how the export of the resulting glycine- and taurine- conjugated bile acids is accomplished.

Peroxisomal β-oxidation may play a role in the degradation of certain prostaglandins, thromboxanes and leukotrienes. It has been shown that some of these compounds accumulate in Zellweger patients, and in some cases that the levels of their β-oxidation products are reduced in these patients [80, 81]. Furthermore, purified peroxisomal preparations were shown to be capable of degrading specific leukotrienes [80]. In particular, peroxisomal involvement has been suggested in the case of prostaglandins F, [82], and E, [83], leukotrienes LTB, LTE, and N-acetyl-LTE, [81] and thromboxane B, [84]. Ferdinandusse et al [85] reported that the levels of LTB, LTE, and their oxidation products secreted in the urine of XALD patients is normal, suggesting that the ABC transporter ALDP is not essential for this pathway. No further data has been published that can provide insight in the transport processes involved in this metabolic pathway.

Lastly, some xenobiotics with an aliphatic or carboxyl side chain appear to be preferentially oxidized
by the peroxisomal β-oxidation system [86-90]. Commonly, β-oxidation ceases after the acyl-group has been shortened by several cycles of β-oxidation. No reports are available on how the import of xenobiotics and export of degradation products across the peroxisomal membrane is accomplished.

1.4 α-oxidation

β-oxidation is hindered by the presence of a methyl-group at the third position of some fatty acids. The most studied fatty acid of this kind is phytanic acid (3,7,11,15-tetramethylhexadecanoic acid). To allow these fatty acids to enter β-oxidation, the carbon chain may be shortened by one carbon atom via α-oxidation. The result is that the methyl group is then present at the 2-position, which poses no problem to further degradation by the peroxisomal or mitochondrial β-oxidation system. A β-oxidation cycle of a 2-methyl fatty acid releases a propionyl-CoA unit rather than acetyl-CoA.

A number of cofactors are required for α-oxidation. Phytanoyl-CoA hydroxylase catalyzes a dioxygenase reaction that requires 2-ketoglutarate, oxygen, Fe$^{2+}$ and ascorbate as cofactors. The reaction yields CO$_2$ and succinate as products. In the context of transport requirements, it should be noted that 2-ketoglutarate, Fe$^{2+}$, ascorbate and succinate are not normally able to permeate a lipid bilayer, while it is well established that the neutral compounds CO$_2$ and O$_2$ can diffuse rapidly across such membranes.

Our own experiments (chapters 5 and 6 of this thesis) suggest that 2-ketoglutarate and succinate can pass the peroxisomal membrane by means of hitherto unidentified transporter(s). No data is currently available on how the other cofactors, Fe$^{2+}$ and ascorbate, are acquired. In view of the ability of the protein import machinery to import large, fully folded proteins, the possibility exists that some cofactors are tightly bound by the protein during the folding process in the cytosol and subsequently co-imported.

The subsequent reaction, catalyzed by 2-hydroxyphytanoyl-CoA lyase, produces formyl-CoA, but this is rapidly converted into CO$_2$ and free CoASH. The fate of CoASH in the peroxisomal matrix is unknown. Recycling of free CoASH as a substrate for peroxisomal acyl-CoA synthetase would yield a stochiometrically 'closed system' for the α-oxidation of phytic acid (and perhaps other α-oxidation substrates). However, studies on the activation of phytic acid do not firmly establish which enzyme(s) are involved. It was shown by Watkins et al [93] that the peroxisomal long-chain acyl-CoA synthetase identified in rat liver is able to activate phytanic acid. However, Singh et al [94], found a microsomal and mitochondrial localization for phytanoyl-CoA ligase activity in rats, but a peroxisomal localization in human tissue. Obviously, future research will have to indicate in which subcellular compartment phytic acid activation is accomplished. This would be expected to yield insight into the nature of the substrate transported into the peroxisome.

1.5 Ether-phospholipid biosynthesis

Some of the enzymes required for the biosynthesis of ether-phospholipids in mammals are located in the peroxisomal matrix [95]. The subcellular distribution of the enzymes involved in this pathway suggests that a fatty alcohol must pass the peroxisomal membrane. Furthermore, dihydroxyacetone phosphate (DHAP) must be either imported into the peroxisome or formed by peroxisomal glycerol-3-phosphate dehydrogenase. In the last case, glycerol-3-phosphate (G3P) would need to be imported into the peroxisome. Preliminary experiments in which the uptake of radiolabeled G3P by proteoliposomes containing peroxisomal membrane proteins was measured indicated that G3P is probably able to pass the peroxisomal membrane (unpublished results). Subsequent reactions in this pathway take place at
the endoplasmatic reticulum, suggesting that alkyl-DHAP is exported from the peroxisomes.

1.6 Metabolite transport
De Duve and co-workers already noted the unusual equilibrium density of peroxisomes in sucrose density gradients [2]. They inferred that the peroxisomal membrane is permeable to sucrose, which agreed well with their failed attempts to observe structure-linked latency for some of the then known peroxisomal enzymes: urate oxidase, D-amino acid oxidase and L-2-hydroxyacid oxidase. Although they did observe structure-linked latency in the case of catalase, this was explained as being due to the first-order kinetics of catalase and the high concentration of catalase in the peroxisome [2]. Several subsequent studies also seemed to indicate that the membrane of isolated peroxisomes is permeable to small molecules.

More recently however, it has been suggested that the permeability properties of peroxisomes in-vitro are fundamentally different from those in-vivo. The difference may perhaps be caused by damage inflicted during the isolation procedure. In support of this notion, the phenotype of certain gene disruption mutants in S. cerevisiae strongly suggests the requirement of shuttle systems for the transport of reducing equivalents [96-98] and acetyl- and propionyl- units [99] across the peroxisomal membrane. However, these opposing viewpoints need not mutually exclude each other. A third possibility remains, in which the peroxisomal membrane is permeable to specific metabolites. This would necessitate the existence of shuttle systems, as observed, to convert some metabolites (for which the peroxisomal membrane is impermeable) to other compounds that can pass across.

1.6.1 Permeability of the membrane of isolated peroxisomes
As mentioned, de Duve and co-workers already concluded that the membrane of isolated peroxisomes is unusually permeable to small molecular weight solutes, based on the observed equilibrium density of peroxisomes in sucrose gradients, and failed attempts to observed structure-linked latency [2]. Further studies were performed by Van Veldhoven et al [100] who determined the accessible volume of several radiolabeled compounds in a concentrated fraction of purified rat liver peroxisomes. Their observations suggested that the peroxisomal membrane is permeable to sucrose, glucose, urea, methanol, acetate, CoASH, ATP, NAD$^+$ and urate. Large molecular weight compounds, such as inulin (±5 kD) or dextran were found not to be able to access the intraperoxisomal space in-vitro. They postulated the existence of a pore-forming protein, or 'porin'. In a subsequent study by the same group, an attempt was made to purify the putative porin [101]. A partially purified fraction was obtained, which contained a 28 kD and a 22 kD protein. However, the 28 kD protein has never been unequivocally identified. An abundant 22 kD peroxisomal integral membrane protein has been cloned later and studied but its precise function has never been resolved. No further evidence has been reported that suggests that it may contribute to the permeability of the peroxisomal membrane. This protein is discussed further in section 1.7.4. Sutler et al [102] reported the purification of a 31 kD protein from peroxisomes of the yeast Hansenula polymorpha that appears to have pore-forming properties. However, they also did not further identify the protein.

More evidence in support of a peroxisomal channel was generated by patch-clamp and black lipid bilayer experiments. Labarca et al [103] reconstituted rat liver peroxisomal membrane proteins in a planar lipid bilayer and detected two channels, the first one being a voltage-sensitive, cation-selective channel with a $P_v/P_o$ of approximately 4 and two open-states with channel diameters of 1.5 and 3 nm. The other channel appeared to be perfectly anion-selective and also has two open-states with conductances of 50 and 100 pS in 0.1 M KCl.

Using the patch-clamp technique, Lemmens et al [104] also detected channels in peroxisomal
membranes isolated from rat liver and the yeast *H. polymorpha*. They reported a conductance of 420 pS for the rat channel and 360 pS for the *H. polymorpha* channel, both in 100 mM KCl. Furthermore, cation specificity was observed for both channels, as evidenced by a $P_+/P_-$ of about 3 for the rat liver channel and 6 for the *H. polymorpha* channel. Both channels were found to exhibit a slight voltage sensitivity. A few notable differences were observed between the two channels. Firstly, solubilization and reconstitution of the rat liver channel in artificial liposomes was found to markedly alter its properties, while the reconstituted *H. polymorpha* protein was electrophysiologically indistinguishable from the protein in native membranes. Secondly, the *H. polymorpha* channel was found to exhibit sensitivity to calcium, the channel being open at high calcium concentrations.

More recently, Reumann *et al* [105, 106] reported detecting a channel in spinach leaf peroxisomes, using planar lipid bilayer techniques. They found a conductance of 350 pS in 1 M KCl (100 pS in 0.1M), and a marked anion-selectivity. Intriguingly, they observed that the permeation of chloride through the channel could be blocked by specific anions. A particularly high affinity for C$_4$-dicarboxylic anions was observed. This led them to suggest that the detected channel has notable substrate specificity. The observed substrate specificity agrees strikingly well with our current understanding of the malate / oxaloacetate and isocitrate / α-ketoglutarate shuttles (sections 1.6.3 and 1.6.4). The estimated channel diameter of around 0.5 nm is too small to allow the passage of large cofactors, such as NADH, CoASH or ATP. They also reported the presence of a similar channel in castor bean glyoxysomes [107]. Unfortunately, the channel was never identified (personal communication).

### 1.6.2 pH of the peroxisomal lumen in vivo

Several groups have reported on the existence of a pH gradient across the peroxisomal membrane *in-vivo*, suggesting a restricted permeability of the peroxisomal membrane. However, the observed intraperoxisomal pH varies considerably. Early work by Nicolay *et al* [108] using $^3$P-NMR suggested an acidic pH for the peroxisomal compartment of methanol-grown cells of the yeast *H. polymorpha*. Their results were supported by Waterham *et al* [109] using DAMP-labelling of spheroplasts. More recently, Lasorsa *et al* [110] also reported observing a low pH in the peroxisomes of *S. cerevisiae* cells grown on oleic acid, using a pH-sensitive fluorescent probe that is targeted to the peroxisome.

In contrast, Dansen *et al* [111] also used peroxisomaly targeted fluorescent probes to measure the pH of human fibroblasts, which they reported to be approximately 8.2

Similarly, van Roermund *et al* [112] observed an alkaline pH in *S. cerevisiae* cells grown on oleic acid / glycerol medium. They provide evidence to suggest that the observed pH gradient may assist in the import of fatty acids via the free-fatty acid route (section 1.6.5). Obviously, future research is required to clarify this matter.

### 1.6.3 Reducing equivalents: NAD$^+$/NADH

Persuasive evidence that the peroxisomal membrane is impermeable to NAD$^+$ and NADH *in-vivo* has come from studies in which β-oxidation was measured in certain *S. cerevisiae* gene disruption mutants. The third step in β-oxidation is a 3-hydroxyacyl-CoA dehydrogenase reaction, in which NAD$^+$ serves as a cofactor (figure 1). Van Roermund *et al* observed that disruption of the gene encoding peroxisomal malate dehydrogenase (*MDH3*) blocks β-oxidation [97], whereas β-oxidation was found to proceed at a normal rate in cell lysates prepared from *MDH3Δ* cells. Moreover, they found an accumulation of the 3-hydroxyacyl-CoA β-oxidation intermediate, which indicates that the defect lies at the level of the NAD$^+$ - dependent step in the β-oxidation pathway. Taken together, these results strongly suggest that NAD$^+$ is not able to permeate the peroxisomal membrane, and that NAD$^+$ is regenerated by malate dehydrogenase to meet the demand of β-oxidation.

Presumably, the substrates and products of Mdh3p, i.e. malate and oxaloacetate, are shuttled across
the peroxisomal membrane (Figure 2A). Evidence that indicates that both compounds can pass across the peroxisomal membrane is presented in chapter 6 of this thesis. A similar shuttle operates in mitochondria (Figure 2B). However, in cases where the mitochondrial membrane is impermeable to oxaloacetate, such as for instance in rat heart [113], transport appears to occur in the form of aspartate instead which is generated by a mitochondrial glutamate:aspartate aminotransferase (GOT). After translocation, oxaloacetate is regenerated by cytosolic GOT. Interestingly, it has been demonstrated that peroxisomes of *S. cerevisiae* and various plant species [114-116] also contain aspartate aminotransferase, suggesting that a similar malate / aspartate shuttle may operate in peroxisomes. However, *S. cerevisiae* gene disruption mutants lacking the peroxisomal aspartate aminotransferase (Aat2p) do not exhibit a β-oxidation defect [116]. In addition, expression of Aat2p is not enhanced by growth on oleate containing medium [116], in contrast to Mdh3p and most other enzymes that perform a metabolic function required for fatty acid β-oxidation in *S. cerevisiae*. Aat2p does therefore not appear to play an essential role in the malate / oxaloacetate redox shuttle.

Mammalian peroxisomes do not contain malate dehydrogenase. However, alternative pathways can be envisioned that would be able to provide a supply of NAD⁺. The presence of NAD-linked glycerol-3-

![Figure 2: Schematic representation depicting the regeneration of NAD⁺ in peroxisomes (A) and in mitochondria (B). Abbreviations used: MDH, malate dehydrogenase; AAT, glutamate:aspartate aminotransferase](image-url)
phosphate dehydrogenase (G3PDH) has been demonstrated in peroxisomes of mammalian cells [117] and in the glycosomes of the trypanosome T. brucei [118]. Therefore, a shuttle based on G3PDH is also conceivable (figure 3A). Although our own experiments do indicate that the peroxisomal membrane is permeable to glycerol-3-phosphate (unpublished observations), no experimental evidence is available to indicate whether this shuttle is responsible for the transfer of reducing equivalents in-vivo.

Alternatively, a shuttle can be postulated based upon peroxisomal lactate dehydrogenase (figure 3B), which has been shown to be present in the peroxisomal matrix despite the fact that it does not possess a known peroxisomal targeting signal [39]. McClelland et al [119, 120] found a partial peroxisomal localization of the monocarboxylate transporters MCT1 and MCT2 in rat liver. They reported that the β-oxidation of a purified peroxisomal fraction in-vitro is stimulated when pyruvate is supplied, and that this stimulation is significantly inhibited by the addition of α-cyano-4-hydroxycinnamate, which is an inhibitor of MCT. Osmundsen et al [121] also found a slight stimulation of β-oxidation upon addition of pyruvate to isolated rat liver peroxisomes in-vitro.

The MCT family of transporters is thought to mediate the symport of protons and monocarboxylates across a membrane. However, some of our own findings indicate that lactate is able to diffuse across a lipid bilayer at a significant rate even without the aid of a transporter in its protonated, neutral form (chapter 5 of this thesis). Furthermore, this diffusion of lactate becomes directional when a ΔpH is applied, since a higher concentration of protonated lactate then exists at the acidic side of the membrane. This was readily demonstrated by monitoring the uptake of radiolabeled lactate into liposomes when a pH gradient was established across the membrane. The lactate uptake could be inhibited strongly by the addition of nigericin, which dissipates the pH gradient. We found that pyruvate behaves similarly. These observations indicate that a transporter protein might not necessarily be required to mediate the transfer of lactate and pyruvate. Whether the rate of diffusion is sufficient to adequately supply NAD⁺ for β-oxidation in-vivo remains undetermined. However, it seems that caution should be observed in the interpretation of the results of in-vitro experiments with isolated peroxisomes, such as reported by Osmundsen et al [121] and McClelland et al [120].

1.6.4 Reducing equivalents: NAD⁺/NADPH

As discussed, the peroxisomal β-oxidation of unsaturated fatty acids with a double bond at an even position requires the activity of NADPH-dependent 2,4-dienoyl-CoA reductase. Studies of S.

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**Figure 3**: Alternative shuttles for the regeneration of NAD⁺ in mammalian peroxisomes. Abbreviations used: G3PDH, glycerol-3-phosphate dehydrogenase; LDH, lactate dehydrogenase.
Chapter 1

*S. cerevisiae* gene disruption mutants have yielded compelling evidence that the NADPH required for this reaction is supplied by peroxisomal isocitrate dehydrogenase (Idp3p).

Van Roermund *et al* [96] and Henke *et al* [98] demonstrated that *S. cerevisiae* IDP3Δ cells are deficient in the β-oxidation of fatty acids with double bonds at an even position, such as petroselenic acid (C18:1n-6), linoleic acid (C18:2n-6) and arachidonic acid (C20:4n-3). Oleic acid β-oxidation proceeds normally in these knockouts, indicating that the defect is specific for fatty acids with a double bond at an even position. Furthermore, van Roermund *et al* [96] found that IDP3Δ cells accumulate the 2,4-dienoyl-CoA precursor of the NADPH-dependent reaction.

These findings pointed to the existence of an isocitrate / 2-ketoglutarate shuttle in peroxisomes that is essential for the regeneration of NADPH. Mammalian peroxisomes also contain isocitrate dehydrogenase [122], indicating that a similar shuttle could potentially function in mammals. A peroxisomal transporter for isocitrate and 2-ketoglutarate has not been identified to date. However, evidence is put forward in chapters 3 and 5 of this thesis that these compounds are capable of passing the peroxisomal membrane.

### 1.6.5 Fatty acid import

Most of our current understanding regarding the import of fatty acids into the peroxisomal matrix comes from studies of the yeast *S. cerevisiae*. Peroxisomes in this organism do not contain carnitine acyltransferase, suggesting that the uptake of fatty acids by the organelle does not take place in the form of a carnitine ester as in mitochondria. Mammalian peroxisomes do contain carnitine acyltransferase (COT), but C26:0-CoA and THCA-CoA are not converted into the respective carnitine esters by cytosolic CPT1 [123]. In agreement, Jakobs *et al* [124] reported that the addition of POCA, an inhibitor of carnitine palmitoyltransferase I to human fibroblasts had virtually no effect on C26:0 β-oxidation, while it completely blocked C16:0 β-oxidation.

It is currently believed that fatty acids can enter the peroxisome either as free fatty acid or directly as a CoA ester. The peroxisomal membrane in *S. cerevisiae* contains two members of the superfamily of ATP-binding cassette transporters, Pxa1p and Pxa2p [125, 126] (discussed in more detail in section 1.7.1). Hettema *et al* [127] found that the β-oxidation of oleic acid (C18:1) is impaired in PXA1 and PXA2 gene disruption mutants, but normal in cell lysates. This suggested a function in a transport process required for peroxisomal β-oxidation. Moreover, since the β-oxidation of lauric acid (C12:0) was found to be completely normal in these knockouts, it was concluded that the defect occurs upstream of the β-oxidation process, presumably at the level of fatty acid import. These results suggested the existence of at least two distinct pathways for the import of fatty acids: one Pxa1p / Pxa2p independent, which is utilized by lauric acid, and another Pxa1p / Pxa2p dependent pathway, by which oleic acid is imported. It was also reported that, conversely, the β-oxidation of lauric acid is impaired in a gene disruption mutant which lacks Faa2p, a peroxisomal acyl-CoA synthetase, while oleic acid is normally β-oxidized by these cells [127, 128]. This led to a model in which lauric acid is imported as a free fatty acid that requires activation to its CoA ester by Faa2p, while oleic acid is activated in the extraperoxisomal space and imported as a CoA ester directly. This model was further consolidated by the observation that redirection of Faa2p to the cytosol (by disruption of its PTS1 targeting signal) causes lauric acid β-oxidation to become fully Pxa1p / Pxa2p dependent [127].

Most fatty acids appear to be imported through both pathways to various extents. Two factors are probably decisive for which pathway is utilized by a specific fatty acid. Firstly, the physiochemical properties of a fatty acid, in particular its solubility in a nonpolar solvent, play an important role. It is well known that the permeability coefficient of small molecules is strongly correlated with their solubility in nonpolar solvents relative to their solubility in water. Secondly, the substrate specificity and subcellular localization of the acyl-CoA synthetases present...
in a cell is a determining factor. As mentioned, expression of a cytosolic variant of the acyl-CoA synthetase Faa2p in *S. cerevisiae* shifts peroxisomal C12:0 import almost entirely to the Pxa1p / Pxa2p dependent pathway.

The described model appears to successfully explain the translocation of most fatty acids. However, a few results have been reported that seem inconsistent with this model, suggesting that it may not be entirely correct.

Recently, Fulda *et al.* [129] reported a study on the involvement of the peroxisomal full ABC-transporter Pxa1p and two peroxisomal long-chain acyl-CoA synthetases (LACS6 and LACS7) in the import of fatty acids into peroxisomes of *Arabidopsis thaliana*. Surprisingly, it was found that a β-oxidation defect occurs in plants lacking Pxa1p, but also in double knockout mutants lacking both LACS6 and LACS7. An acyl-CoA synthetase single-knockout (in which at least one peroxisomal acyl-CoA synthetase remains) did not exhibit an observable phenotype. This suggested to the authors that the import of fatty acids for β-oxidation in this plant does not occur via two independent pathways but requires the concerted action of both Pxa1p and peroxisomal acyl-CoA synthetases. They propose a modified model where Pxa1p hydrolyzes acyl-CoA and releases a free fatty acid into the peroxisomal matrix which then needs to be reactivated by a peroxisomal LACS (figure 4).

In this context, it is of interest to note that *A. thaliana* Pxa1p is somewhat unusual when compared to its yeast and mammalian orthologues. While the known peroxisomal ABC transporters in other organisms are half-ABC transporters (which are thought to dimerize to produce a functional transporter) the *A. thaliana* Pxa1p is a full ABC-transporter (which is believed to operate as a monomer). The significance of this marked difference is unknown, but clearly the situation in *A. thaliana* is not necessarily representative for other organisms.

Alternatively, the results observed by Fulda *et al.* [129] may also be explained if *A. thaliana* Pxa1p would be involved in the transport of a cofactor required to sustain the activity of peroxisomal LACS (such as CoA, adenine nucleotides or phosphate). More insight in the actual substrate of *A. thaliana* Pxa1p is clearly required to comprehend the significance of the observed results.

The model of Fulda *et al.* [129] cannot be extended to *S. cerevisiae* without further modifications to our current understanding of peroxisomal fatty acid import in this organism. The transport of adenine nucleotides across the peroxisomal membrane in *S. cerevisiae* is performed by Ant1p, a peroxisomally localized member of the Mitochondrial Carrier Family (MCF / SLC25) [130, 131]. Consistent with the original model for fatty acid import, an *ANT1Δ* mutant exhibits a similar phenotype as the *FAA2Δ* mutant. This would be explained by the current model as an inability of Faa2p to activate fatty acids intraperoxisomally due to a lack of adenine nucleotides. The β-oxidation of fatty acids that

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**Figure 4:** Models for the import of fatty acids into the peroxisome. (A) Current model; (B) Model as proposed by Fulda *et al.* [129]. Abbreviations used: FFA, free fatty acid
utilize the Pxa1p / Pxa2p acyl-CoA import pathway is unaffected in ANTIΔ cells, suggesting that an imported fatty acid is released into the lumen of the peroxisome as a CoA ester, abolishing the need for intraperoxisomal activation, and not as a free fatty acid as suggested by Fulda et al [129].

1.6.6 Import of other β-oxidation substrates

Jedlitschky et al [132] observed that isolated peroxisomes are able to degrade the leukotriene -hydroxy-N-acetyl-LTE₄ (in the presence of appropriate cofactors) only when a microsomal fraction was added to provide an acyl-CoA synthetase activity. Schepers et al [83] made a similar observation using prostaglandine E₂. This may imply that these compounds, and perhaps related compounds, are activated in microsomes and subsequently imported into the peroxisome. How the transport of the CoA esters across the peroxisomal membrane is accomplished remains unclear. Ferdinandusse et al [85] have reported that the levels of leukotrienes LTB₄, LTE₄, and their oxidation products are normal in the urine of XALD patients. Similarly, Mayatepek et al [133] observed a normal leukotriene profile in the bile of XALD patients, suggesting that the ABC transporter ALDP is not required for the import of these compounds into the peroxisome.

The activation of bile acid precursors to their respective CoA esters is required prior to β-oxidation. Mihalik et al [134] reported that peroxisomal very-long-chain acyl-CoA synthetase is at least capable of this reaction. In contrast, both Prydz et al [135] and Koibuchi et al [136] found that THCA-CoA synthetase activity is localized exclusively to microsomes. Hence, whether bile acid precursors are imported into peroxisomes as CoA esters remains unclear.

1.6.7 Export of β-oxidation products

Depending on the substrate entering β-oxidation, several products may emerge. Degradation of a carboxyl chain yields acetyl-CoA and, if a 2-methyl side chain is present, propionyl-CoA. Degradation of some substrates yields a residual compound that cannot be β-oxidized further, such as for example in the case of certain xenobiotics and leukotrienes, etc. For other substrates, the peroxisomal β-oxidation system functions as part of a biosynthetic pathway, for example in the case of bile acid intermediates and DHA. Although further β-oxidation would theoretically be possible, this may not be desired. In these cases, β-oxidation can be terminated after a specific cycle and the ensuing product exported from the peroxisome. As discussed, the peroxisomal β-oxidation in mammals does not proceed to completion even in the case of simple fatty acids. Instead, residual medium- and short-chain fatty acids remain that are transferred to the mitochondria for continued β-oxidation.

1.6.8 Acetyl- and propionyl-CoA

Van Roermund et al [97] used the model organism S. cerevisiae to study the transfer of acetyl- and propionyl units from peroxisomes to mitochondria. Based upon knowledge of the peroxisomal protein content, two pathways could be envisioned to provide an export route for acetyl- units from the peroxisome: (i) by entry into the glyoxylate cycle via peroxisomal citrate synthetase (Cit2p), or (ii) by conversion into a carnitine ester by peroxisomal carnitine acetyltransferase (Cat2p). They observed that a double knockout lacking both the CIT2 and CAT2 genes exhibits a β-oxidation defect. In contrast, a normal β-oxidation activity was found in cell lysates of these cells, which suggested that the defect might lie at the level of membrane translocation. The single gene disruption mutants CIT2Δ and CAT2Δ each display only a slight reduction in the rate of β-oxidation, indicating that both proposed routes can contribute significantly to the export of acetyl-CoA. These results strongly suggest that acetyl- and propionyl-CoA are unable to traverse the peroxisomal membrane, but are converted into carnitine esters prior to transport out of the peroxisome. It should be noted that acetyl-units may also leave the peroxisome through the glyoxylate cycle.
Attempting to obtain more insight in the transfer of propionyl units from peroxisomes to mitochondria in man, Jakobs et al [137] supplied \([1-^{14}C]\) radiolabeled pristanic acid to fibroblasts of patients with a defect in the gene encoding mitochondrial carnitine / acylcarnitine translocase (CACT) or carnitine palmitoyltransferase 2 (CPT2). They observed that \(^{14}CO_2\) formation was completely deficient in the CACT deficient cells, but not in the CPT2 deficient cells. This suggested that CACT participates in the transfer of propionyl- units into the mitochondrion where they can be oxidized to yield \(^{14}CO_2\).

Although the described studies provide evidence that acetyl- and propionyl-CoA units are exported from the peroxisome as carnitine esters in both yeast and man and that acetyl units may also leave the peroxisome through the glyoxylate cycle in yeast, they do not provide insight into how the actual translocation across the peroxisomal membrane occurs.

Van Roermund et al [99] performed a genetic screen in S. cerevisiae designed to identify gene disruption mutants that are specifically impaired in the Cat2p-dependent route for acetyl units from peroxisome to mitochondrion. Although they identified several genes that may be expected to contribute to this pathway, such as the mitochondrial carnitine:acylcarnitine carrier (Crc1p), a previously unidentified plasma membrane carnitine transporter (Agp2p), this screen did not yield any candidate for a peroxisomal acetyl carnitine transporter. Possibly, the phenotype of a gene disruption mutant lacking the peroxisomal carnitine transporter is not immediately recognisable, e.g. it may be lethal, or it may lead to abnormal peroxisomal morphology causing it to be classified as a PEX gene. Alternatively, multiple transporters may exist in the peroxisomal membrane for acetyl carnitine, so that disruption of a single gene does not produce a phenotype.

The distribution of glyoxylate cycle enzymes between the cytosol and peroxisome suggests the translocation of glyoxylate cycle intermediates across the peroxisomal membrane. Acetyl- units produced by \(\beta\)-oxidation can enter the glyoxylate cycle on the peroxisomal side via Cit2p, which results in the release of a succinate moiety on the cytosolic side by cytosolic isocitrate lyase. Succinate can subsequently enter the mitochondrion via the mitochondrial fumarate / succinate transporter Acr1p for further oxidation, ultimately yielding \(^{14}CO_2\) and \(H_2O\).

1.7 Peroxisomal transporters

1.7.1 ABC-transporters

The superfamily of ATP-binding cassette (ABC) transporters is a large family of integral membrane proteins that mediate the transport of a variety of substrates. A number of recent reviews are available on this fascinating superfamily of proteins [138-141]. In particular, the role of ABC-transporters in the export of xenobiotics has attracted considerable interest because it has been found to constitute an important mechanism behind the drug resistance of some cancer cells. Increasing numbers of human disorders are being discovered that are caused by mutations in ABC transporters [141, 142], such as cystic fibrosis (caused by mutations in CFTR), Stargardt’s disease (ABCR), X-linked adrenoleukodystrophy (ABCD1), Dubin-Johnson syndrome (MRP2), etc.

ABC-transporters are composed of 4 domains: two membrane-spanning domains (TM), that presumably constitute a translocation channel, and two nucleotide binding domains (NBD). The ATP binding domain harbours Walker A and Walker B motifs, and a unique motif of unknown function called the ‘C motif’ or ‘LSGGQ motif’. In addition, bacterial ABC-transporters sometimes also involve a soluble substrate binding component, for example in the case of the histidine [143] and maltose transporters [144]. The individual domains may be integrated in a single polypeptide or distributed over multiple polypeptides. The two most commonly encountered forms are (i) a single polypeptide containing both transmembrane (TM) and nucleotide-binding domains (NBD), and (ii) two polypeptides each containing a single TM and NBD. The first type is known as a full ABC-transporter.
the second is called a half ABC-transporter. Most probably, half-ABC-transporters dimerize to produce a functional transporter. Well studied examples of human half-ABC-transporters are the intestinal plant-sterol transporter ABCG5 / ABCG8 [145] and endoplasmatic reticulum antigenic peptide transporter TAP1 / TAP2 [146]. Interestingly, members of the ABCG subfamily identified in Drosophila eye pigment cells heterodimerize in different combinations to yield functional transporters with a different substrate specificity [147].

The mechanism of operation of ABC-transporters has been studied intensively. Although it appears that most members of the superfamily are active pumps that employ the energy of ATP hydrolysis to allow the translocation of substrates against a steep concentration gradient, this is not always the case. Notable exceptions in this respect seem to be for instance CFTR, which is currently believed to be a nucleotide-regulated passive chloride channel [148, 149], and the sulfonylurea receptor SUR1 / SUR2 which is thought to act as regulator for an ATP-sensitive potassium channel [150]. Both are the subject of continued investigations because of their significance in human disease (cystic fibrosis and diabetes respectively).

Several ABC-transporters have been identified in peroxisomal membranes: four half-transporters in mammals (ALDP / ABCD1, ALDR / ABCD2, PMP70 / ABCD3 and PMP69 / ABCD4), two half-transporters in S. cerevisiae (Pxa1p and Pxa2p), and a single full ABC-transporter in A. thaliana (PXA1 / PED3 / COMATOSE / CTS) [151]. Their functions have not been established unequivocally. As discussed in section 1.7.1, Pxa1p and Pxa2p are thought to function in the translocation of acyl-CoA into the peroxisomal matrix. ALDP is believed to perform the same function in mammalian peroxisomes. The accumulation of dicarboxylic acids in PMP70 knock-out mice suggests that this protein may be involved in the import of certain fatty acids into the peroxisomal matrix. Very little information is available that can provide insight in the function of ALDR or PMP69. Some suggestive evidence exists to indicate that there might be some functional redundancy between ALDP and ALDR. Several groups reported that the addition of compounds that induce the expression of ALDR to cultured XALD fibroblasts is accompanied by an increase in VLCFA β-oxidation [152-154].

Considerable effort has been made to reveal which of the half-ABC-transporters dimerize, with conflicting results. Shani et al [155] concluded that the S. cerevisiae transporters Pxa1p and Pxa2p heterodimerize based upon co-immunoprecipitation experiments. Their conclusion is further consolidated by the observation that disruption of the PXA2 gene has considerable effect on the stability of Pxa1p. Conversely, the stability of Pxa2p is unaltered by disruption of the PXA1 gene. Their results suggest a physical interaction between the proteins.

The situation is more complex in mammals due to the presence of at least four half-transporters. Liu et al [156] used the yeast two-hybrid system to investigate the dimerization of the carboxyl-terminal halves of ALDP, ALDR and PMP70. In addition, they employed immunoprecipitation to obtain data on the situation of the native proteins. Based on these results, the authors concluded that both hom- and heterodimerization in various combinations occur.

Guimaraes et al [157] recently purified mouse liver PMP70 and ALDP to apparent homogeneity in the presence of the mild detergent digitonin. They found no other proteins associated with the purified proteins, which led them to conclude that these transporters predominantly exist as homodimers in the peroxisomal membrane.

Berger et al [158] examined the tissue distribution of each of the four peroxisomal ABC-transporters in various mouse tissues. They observed a different tissue distribution for each member, and distinct temporal expression levels during brain development, arguing against the obligatory formation of specific heterodimers. The spatial distribution of PMP70 and ALDP mRNA levels in rat brain was investigated by Pollard et al [159]. Similarly, they observed no correlation in expression levels. Although most ABC-transporters seem to utilize the energy from ATP-hydrolysis to drive transport,
this mechanism is not universal. The examples of CFTR and SUR1/SUR2 have been mentioned already.

Several reports have been published suggesting that the peroxisomal ABC-transporters may be capable of ATP hydrolysis. Gartner et al. [160] purified the NBD of human ALDP in fusion with maltose binding protein, and showed that the isolated domain is capable of ATP-hydrolysis. They also found that mutations identified in XALD patients disrupt ATP-hydrolysis. Similarly, Roerig et al. [161] purified the NBD’s of human PMP70 and ALDP and arrived at the same conclusion. However, whether the activity of the NBD isolated in this manner may be extrapolated to the activity of the full transporter is uncertain. Several examples exist that indicate that the ATP hydrolysis properties of ABC-transporters change when the protein is removed from its native environment in a lipid membrane [162]. In fact, even the lipid composition of the membrane can affect ATP hydrolysis and transport activity considerably [162]. Therefore, Tanaka et al. [163] used photoaffinity labelling and immunoprecipitation to show that native rat-liver PMP70 and ALDP in isolated peroxisomes is probably also able to hydrolyse ATP.

The human disorder X-linked adrenoleukodystrophy is caused by mutations in the ABCD1 gene [164]. Biochemically, these patients are characterized by elevated levels of very-long-chain fatty acids. The clinical presentation of XALD patients varies considerably, even among patients with the same mutation in ABCD1 [165], suggesting a modulating factor which has not been identified. The half-transporter nature of ALDP suggests that perhaps variations in the dimerization partner of ALDP are responsible, but no relation to mutations or polymorphisms in the other peroxisomal ABC-transporters has been found.

No human disease has been linked to mutations in the other ABC-transporters. Previous reports that mutations in PMP70 cause a form of Zellweger syndrome [166] turned out to be in error [167]. Strong evidence that disruption of PMP70 does not lead to Zellweger syndrome is provided by the observation that peroxisomes in PMP70 knock-out mice appear normal.

1.7.2 Ant 1p / PMP34

In 1990, McCammon et al. [168] identified a gene in the methylotrophic yeast Candida boidinii that codes for a peroxisomal membrane protein of 47 kD (PMP47). Although its function was unclear at the time, it was soon recognized on the basis of primary sequency homology as a member of the Mitochondrial Carrier Family (MCF / SLC25) [169]. Since then, orthologues have been identified in A. thaliana (PMP38) [170], humans (PMP34) [171], and S. cerevisiae (Ypr128c / ANTI) [130].

The first indication of its function came from a study by Nakagawa et al. [172], who showed that the C. boidinii protein is required for the β-oxidation of lauric acid (C12:0), but not for the β-oxidation of oleic acid (C18:1). As discussed in section 1.6.5, medium chain fatty acids, such as lauric acid, are activated in the peroxisomal matrix by Faa2p, while oleic acid is most likely imported into the peroxisome as a CoA ester. Their results suggested that the protein is involved in the transport of a factor that is required for the intraperoxisomal activation of fatty acids. Subsequently, Van Roermund et al. [130] showed that the S. cerevisiae protein is most likely involved in the transport of ATP, using the peroxisomal protein luciferase to detect intraperoxisomal ATP in-vivo. Ultimately, direct evidence that the human and S. cerevisiae proteins are indeed involved in the transport of adenine nucleotides was gained by reconstitution of the purified protein in liposomes, and following the uptake of radiolabeled substrates by these proteoliposomes [131, 173]. Our results with the human protein, PMP34, are described in chapter 2 of this thesis.

Very recently, more detailed studies of the properties of the S. cerevisiae protein suggest that it also participates in generating a pH gradient across the peroxisomal membrane [110, 112]. Lastly, the proper localization of dihydroxyacetone synthetase (DHAS) was found to be disturbed
in methanol-grown *C. boidinii* cells in which PMP47 was disrupted [174]. The localization of other peroxisomal proteins appeared to proceed normally. The role of PMP47 in the peroxisomal localization of DHAS has never been resolved.

### 1.7.3 PEX11

As discussed earlier, our understanding of the function of the peroxins is very limited. The designation ‘PEX’ was often given to genes and when aberrant peroxisome morphology was observed in a gene-disruption mutant. The possibility remains that some of the peroxins primarily serve a role in metabolism. In support of this possibility, abnormal morphology is also observed in oleate-grown *S. cerevisiae* gene disruption mutants lacking for instance acyl-CoA oxidase (Poxlp), acyl-CoA Thiolase (Pox3p) or the Adenine Nucleotide Carrier (Antlp) (unpublished results).

The role of Pex11p is debated. All reports seem to agree that disruption of the PEX11 gene leads to a ‘giant peroxisome’ phenotype in which a reduced number of enlarged peroxisomes are formed. Conversely, overexpression results in an increased number of peroxisomes of reduced size [175-177].

Van Roermund *et al* [178] suggested that Pex11p primarily performs a metabolic function in the degradation of medium-chain fatty acids (MCFA) in *S. cerevisiae*, and that the observed morphology is a consequence of the metabolic defect. In contrast, Li *et al* [179, 180] forwarded evidence that the defect lies at the level of peroxisomal proliferation, at least in mice. However, the situation is complicated by the existence of three PEX11 isoforms in humans and mice, PEX11α, PEX11β and PEX11γ. Recently, two additional proteins with extensive homology to Pex11p have been identified in *S. cerevisiae* as well, named Pex25p and Pex27p [181]. Rottensteiner *et al* [181] have proposed a role in biogenesis and proliferation for these proteins, based mainly on the finding that peroxisomal morphology and protein import is disturbed in gene disruption mutants. The possibility cannot be ruled out that some of the functions of the individual mammalian PEX11 isoforms are integrated into a single Pex11p protein in *S. cerevisiae*. This is supported by the observation that *S. cerevisiae* Pex11p mutants can be isolated that are (i) deficient in medium-chain fatty acid β-oxidation but have normal proliferation or (ii) that have normal MCFA β-oxidation but exhibit aberrant peroxisomal morphology (C.W.T. van Roermund, personal communication).

### 1.7.4 PMP22 / Mpv17

Early investigations into the constituents of the peroxisomal membrane identified a 22 kD protein as an abundant component [182-184]. Combined, PMP70 and PMP22 constitute roughly half of the protein content of the membrane of rat liver peroxisomes [185]. Several related 22 kD proteins have been identified in various species: rat PMP22 [186], mouse MPV17 [187], mouse PMP22 / PXMP2 [188, 189], mouse M-LP [190], *A. thaliana* PMP22 [191], human PMP22 [69] and *S. cerevisiae* Ylr251p / Sym1p [192].

All PMP22-related proteins are localized to peroxisomes, with the exception of the yeast protein Sym1p which is localized to the mitochondrial inner membrane (unpublished results and [192]). As discussed, van Veldhoven *et al* [101] speculated that a 22 kD protein might posses pore-forming activity, based on the observation that a protein fraction containing a 22 kD PMP and other PMPs was shown to confer sucrose permeability when reconstituted in liposome membranes. However, the 22 kD protein was not further identified, and their preparation contains multiple proteins. Later studies have been performed to gain more insight into the function of the PMP22-related proteins.

Zwacka *et al* [187] describe their results with an MPV17 knock-out mouse. These mice developed
glomerulosclerosis, nephrotic syndrome [187], hypertension [193] and sensineural deafness [194]. Peroxisomal biogenesis appears to be normal, but a reduced level of reactive oxygen species (ROS) was observed. Overexpression of MPV17 in transfected cells resulted in increased intracellular ROS. Consequently, a role in ROS regulation was proposed. In line with this hypothesis, Wagner et al. [195] found altered levels of antioxidant enzymes in these mice. Furthermore, transfection of COS-7 cells with another PMP22-related protein, murine M-LP, was reported to elevate the expression of manganese superoxide dismutase (SOD2) [196]. Reuter et al. [197] found that the expression of matrix metalloproteinase 2 (MMP-2) is increased in the MPV17 mouse strain.

The mitochondrial yeast protein has also been functionally characterized to some extent. SYM1Δ cells were found to exhibit a growth defect on ethanol at elevated temperature [192]. Dysregulation of several ethanol-repressed genes was observed when the cells were cultured at 37 degrees C, compared to wild-type [192]. Curiously, expression of the murine MPV17 in SYM1Δ cells was found to complement the phenotype. Unfortunately, the subcellular localization of MPV17 in these cells was not determined because attempts to produce an epitope-tagged protein were found to inactivate it, as evidenced by the inability of the tagged protein to rescue the mentioned phenotype. The putative S. cerevisiae protein Yor292c also bears significant sequence homology with other PMP22-related proteins. The protein was localized to the vacuole in a large scale localization study [198]. However, in light of the sensitivity of MPV17 to the presence of an epitope-tag, the influence of the rather large GFP fused to Yor292c in this study could have influenced the result. Furthermore, a putative oleate-responsive-element (ORE box) can be identified in its promoter region (approximately 350 bp upstream of the start codon), suggesting a role for this protein in fatty acid metabolism.

1.7.5 PMP24
Reguenga et al. [199] isolated and identified a peroxisomal membrane protein of 24 kD from rat liver. They subsequently identified a human orthologue in a cDNA library. The function of the protein is unknown. However, the protein does posses significant homology to certain bacterial transporters (e.g. 29% identity with a permease from Bacteroides thetaiotaomicron; GI:29341556 ). Recently, Wu et al. [200] identified the gene in a screen for CpG sites that are differentially methylated and transcriptionally silenced in a comparison of two prostate cancer cell lines.

1.7.6 Short calcium-binding MCF member in rabbit (‘Efinal’)
Weber et al. [201] identified a putative calcium-binding member of the Mitochondrial Carrier Family (MCF / SLC25) of solute carriers from a rabbit small intestinal cDNA library, which they named ‘Efinal’. Using immunoelectronmicroscopy with an antibody generated against a fragment of the native protein they found a bimodal peroxisomal / mitochondrial localization. A function of the protein was not established. However, the calcium-binding properties inferred from the presence of four EF-hand motifs in the N-terminus were confirmed. Later studies by others identified similar proteins in other organisms. All consist of a N-terminal domain containing four EF-hand motifs and a C-terminal domain possessing all the characteristics of MCF proteins. The name Short Calcium-Binding Mitochondrial Carrier (SCaMC) has been suggested for members of this subfamily [202]. So far, other SCaMC members identified were reported to localize exclusively to mitochondria [202, 203]. Fiermonte et al. [204] recently characterized three closely related human SCaMC proteins in-vitro and found that they catalyze a calcium-stimulated Mg-ATP / Pi antiport reaction.

1.8 Scope of this thesis
Previous work has convincingly demonstrated that transport of metabolites across the peroxisomal membrane is facilitated by specific transporters. Based on our current understanding of peroxisomal
metabolism, predictions can be made with respect to the nature of the transporters that may exist in the peroxisomal membrane. It then becomes obvious that our understanding of metabolite transport across the peroxisomal membrane is extremely limited. Numerous metabolites are predicted to pass across the peroxisomal membrane, in both directions, whereas currently only an adenine nucleotide carrier and a presumed acyl-CoA transporter have been demonstrated convincingly. Therefore, the aim of the studies described in this thesis was to gain more insight into how the transport of metabolites across the peroxisomal membrane proceeds. Previous studies were hampered by the apparent permeability of the membrane of isolated peroxisomes, which is possibly an artefact of the isolation procedure. Therefore, to be able to detect the presence of transporters we sought alternative methods that do not rely on the isolation of intact organelles. Eventually, this should lead to identification and detailed characterization of the proteins involved in peroxisomal metabolite transport.

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