Function and dysfunction of the mitochondrial and peroxisomal beta-oxidation systems in human cells and their functional interaction

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Citation for published version (APA):
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GENERAL INTRODUCTION

Cells are the fundamental, structural and functional units of living organisms. A cell can constitute an entire organism, as in Protozoa or, it can be one of many cells that are grouped and differentiated into tissues and organs to form a multicellular organism. A cell can be considered as an organism in itself, often very specialized and composed of many elements. Enzymes are one of these elements. Enzymes are used by cells to produce the various types of energy transformation necessary for maintenance of living activities. Eukaryotic cells, as compared to prokaryotic cells, have a complex organization with the characteristic feature of the compartmentation of processes and biochemical pathways within the organelles. Several organelles can be distinguished based on their function and morphology e.g. nuclei, vacuoles, mitochondria, endoplasmic reticulum, ribosomes, Golgi apparatus and microbodies. This thesis deals with the functioning of two of these organelles, the mitochondrion and the peroxisome, in the intact cell and in particular their role in the β-oxidation of fatty acids in human skin fibroblasts.

1.1 Microbodies

Microbodies first discovered by Rhodin [1954], as "spheric or oval bodies" present in the cytosol of mouse proximal kidney tubules, are organelles characterized by a relatively simple morphology. They consist of a proteinaceous matrix which is bounded by a single membrane and carry out a variety of metabolic functions. All eukaryotic cells, with the exception of mature erythrocytes and primitive eukaryotes, which include Microsporae, Archamoebae and Anaxtylae [Cavalier-Smith, 1991], contain microbodies which vary in size, number and metabolic function. In higher eukaryotes, the significance of microbodies is generally dependent on organism, cell type and developmental stage of the tissue or organism.

The name "peroxisomes" was proposed by de Duve [1965] based on the finding of catalase and H₂O₂-producing enzymes in a single organelle. Later, Breidenbach and Beevers [1967] identified an organelle morphologically similar to the peroxisome in germinating plant seeds and called the organelles glyoxysomes based upon the occurrence of several glyoxylate cycle enzymes within this organelle. Another member of the microbody family was discovered by Opperdoes and Borst [1977] in Trypanosomes, which was given the name glycosome since these organelles appeared to contain many enzymes of the glycolytic pathway. Hydrogenosomes, which are involved in carbohydrate metabolism, were also believed to belong to this microbody family [Müller, 1980], but recently it has become clear, by phylogenetic analyses of hydrogenosomal Hsp 70, Hsp 60 and Hsp10, that hydrogenosomes and mitochondria have a common eubacterial ancestor [Bui et al., 1996]. Additional evidence that peroxisomes, glyoxysomes and glycosomes belong to a single family came from studies by Keller and coworkers [Keller et al., 1991] who showed that these organelles make use of related protein import machineries and protein targeting signals, which suggests a common ancestry. Furthermore, functional transformation of glyoxysomes to leaf peroxisomes during greening and vice versa during senescence, has been observed in higher plant cells [Nishimura et al., 1996]. Typically all types of microbody appear to contain a fatty acid β-oxidation system, involving a H₂O₂
producing oxidase in the first step, with the exception of Neurospora crassa [Kionka and Kunau, 1985] where an acyl-CoA dehydrogenase is catalyzing the first step.

1.2 Peroxisomes

Peroxisomes, like mitochondria and plastids, are believed to have arisen as endosymbionts (for review see [Borst, 1986]) but in contrast to mitochondria and plastids they do not contain DNA [Kamiryo et al., 1982]. Furthermore they are bound by a single lipid membrane with a trilaminar appearance and vary in size from 0.1 µm (micro-peroxisomes in brain or fibroblasts) to 0.5-1.5 µm in mammalian liver or kidney [Lazarow and Fujiki, 1985]. They are numerous in liver, kidney and nervous tissue especially in oligodendroglial cells during the active myelination period, suggesting that peroxisomes are important for the formation of myelin [Arnold and Holtzman, 1978]. It has become clear, mainly by the discovery of several inherited metabolic diseases in which peroxisomes dysfunction, that peroxisomes play an important role in a number of metabolic pathways.

1.2.1 Peroxisomes and their metabolic functions

I. Hydrogen peroxide metabolism

Peroxisomes are characterized by their content of hydrogen peroxide producing flavin-oxidases and catalase, which decomposes hydrogen peroxide [de Duve and Baudhuin, 1966]. Catalase, present in the peroxisomal matrix, decomposes hydrogen peroxide either by conversion of 2 molecules of H₂O₂ to 2 molecules of H₂O and 1 molecule of O₂ or, peroxidatically by conversion of 1 molecule H₂O₂ to 2 molecules of H₂O together with the oxidation of another hydrogen donor (i.e. ethanol, methanol, formaldehyde, formate, nitrite) [de Duve and Baudhuin, 1966; Chance et al., 1979]. The types of substrate that are oxidized by the different oxidases include D- and L-amino acids, L-α-hydroxyacids and urate [de Duve and Baudhuin, 1966; de Duve, 1983], polyamines [Hölttä, 1977; Beard et al., 1985], glutaryl-CoA [Vamecq and van Hoof, 1984], hypoxanthine and xanthine [Angermüller et al., 1987], L-pipelicolic acid [Wanders et al., 1988], acyl-CoA esters i.e. straight-chain [Wanders et al., 1990a], 2-methyl-branched-chain fatty acids [Van Veldhoven et al., 1992; Wanders et al., 1992a], and bile acid intermediates di- and trihydroxyprostanic acids [Vanhove et al., 1993b]. Vamecq and coworkers [1993] suggested the presence of another distinct peroxisomal oxidase, valproyl-CoA oxidase in rat liver, supporting the peroxisomal oxidation of valproyl-CoA and its Δ⁴-enoic derivative. Cu,Zn-superoxide dismutase, a metallo enzyme, which catalyses the dismutation of superoxide anion radicals to H₂O₂ and O₂ by the alternate reduction of a copper ion which constitutes the catalytically active redox centre, has also been shown to be present in rat liver peroxisomes [Dhaunsi et al., 1992; Wanders and Denis, 1992]. H₂O₂ metabolism in peroxisomes functions as a disposal of excesses of reducing equivalents, where energy is partly released as heat (possible role in thermogenesis).
II β-Oxidation of fatty acids and other compounds

Peroxisomes play a major role in the β-oxidation of fatty acids, as first demonstrated by Lazarow and de Duve [1976] following earlier findings by Breidenbach and Beevers [1967] in glyoxysomes. It has now become clear that peroxisomal β-oxidation is involved in the chain-shortening of saturated long-chain fatty acids [Osumi et al., 1980] and very-long-chain fatty acids [this thesis, Chapter 2; Jakobs and Wanders, 1991], mono- and poly-unsaturated fatty acids [Hovik and Osmundsen, 1987; Osmundsen et al., 1991], isoprenoid derived branched-chain fatty acids [Wanders et al., 1990a; Van Veldhoven et al., 1992; this thesis, Chapter 3 and 5], medium- and long-chain dicarboxylic acids [Krivlraa and Gregersen, 1986; Suzuiki et al., 1989], prostaglandins F₂α and E₂ [Diczfaly et al., 1987; Schepers et al., 1988], xenobiotics with an acyl-side chain [Yamada et al., 1986] and leukotrienes [Jedlitschy et al., 1991], cholesterol intermediates i.e. 3α,7α,12α-trihydroxy-5β-cholestanolic acid (THCA) and 3α,7α-dihydroxy-5β-cholestanolic acid (DHCA) [Kase et al., 1985 and 1986; Krisans et al., 1985]. The β-oxidation pathway will be discussed in more detail later on in this chapter.

Figure 1. Phytanic acid α-oxidation in human liver peroxisomes [Verhoeven et al., 1997]

III Phytanic acid α-oxidation

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) formed from phytol, present in plant foodstuff, is a branched-chain fatty acid containing a -CH₃ group at the 3-carbon position. Due to this structure phytanic acid first
needs to be decarboxylated yielding pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) before \(\beta\)-oxidation can occur in peroxisomes. For several years the mechanism of phytanic acid \(\alpha\)-oxidation, its subcellular localization and the enzymes involved remained obscure.

Recent studies, however, revealed that 1) \(\alpha\)-oxidation is initiated in peroxisomes [Verhoeven et al., 1998] (figure 1) and 2) phytanic acid is activated towards phytanoyl-CoA, at the peroxisomal membrane prior to import, by either an enzyme distinct from long-chain and very-long-chain acyl-CoA synthetase as observed in human fibroblasts by Pahan and co-workers [Pahan et al., 1993] or by a single enzyme sharing long-chain acyl-CoA and phytanoyl-CoA synthetase activities as reported for rat liver [Watkins et al., 1996]. Once inside the peroxisome phytanoyl-CoA is hydroxylated to 2-hydroxyphytanoyl-CoA by a newly identified enzyme named phytanoyl-CoA hydroxylase. This is a dioxygenase type of enzyme, using 2-oxoglutarate as co-substrate which is converted to succinate whereas Fe\(^{2+}\) and ascorbic acid are required as cofactors. It has been shown to catalyze the first step in phytanic acid \(\alpha\)-oxidation in human [Jansen et al., 1996] and rat liver [Mihalik et al., 1995] peroxisomes. Recently, phytanoyl-CoA hydroxylase has been purified from rat liver peroxisomes and the full-length human cDNA sequence has been identified [Jansen et al., 1997]. Mutations in the phytanoyl-CoA hydroxylase gene constitute the main course of accumulation of phytanic acid in Refsum disease [Jansen et al., 1997]. Finally, pristanal, identified as product of the decarboxylation of 2-hydroxyphytanoyl-CoA, can be further oxidated in a NAD\(^+\) dependent manner producing pristanic acid [Verhoeven et al., 1997].

**IV Etherphospholipid biosynthesis**

Mammalian peroxisomes play an essential role in the biosynthesis of etherphospholipids. This unique class of phospholipids is ubiquitous in nature. In mammals, these phospholipids have an O-alkyl linkage or an \(\alpha\), \(\beta\)-unsaturated O-alkyl linkage at the sn-1 position of the glycerol backbone and can constitute 5-20 \% of tissue phospholipids. They are abundant in nervous tissue, especially myelin.

Etherphospholipid biosynthesis (figure 2) starts with the acylation of dihydroxyacetone phosphate (DHAP) to acyl-dihydroxyacetone-3-phosphat by acyl-CoA:dihydroxyacetonephosphate acyltransferase (DHAP-AT). This enzyme, which only accepts DHAP as substrate and not glycerol-3-phosphate, is a membrane protein with its active site exposed to the peroxisomal matrix [Hardeman and van den Bosch, 1988; 1989]. DHAP-AT, recently purified from guinea pig liver [Webber and Hajra, 1993] and human placenta [Ofman and Wanders, 1994] was found to be indispensable for ether-phospholipid synthesis [Wanders et al., 1992c]. Next, alkyl-dihydroxyacetone phosphate synthase (alkyl-DHAP synthase) introduces the ether bond by replacing the acyl-group by an alkyl-group [Hajra and Bishop, 1982]. Alkyl-DHAP synthase is also found to be membrane-bound facing the matrix and has been purified too from guinea pig liver [Zomer et al., 1993]. Furthermore, the same group has identified the guinea pig [Vet et al., 1997] and the human [Vet et al., 1997a] cDNAs from alkyl-DHAP synthase. The third enzyme, acyl/alkyl dihydroxyacetonephosphate oxidoreductase reduces the keto-group at the C\(_2\) position. The enzyme is found in both peroxisomes and ER, and is membrane bound facing the cytosol [Hajra and Bishop, 1982; Hardeman and van den
The final steps of etherphospholipid biosynthesis take place in the ER [Hajra and Bishop, 1982]. The function of etherphospholipids remains largely unknown, with the exception of platelet-activating factor (PAF) and plasmalogens, which can be formed from alkyl-acyl phospholipids by means of a desaturase [Snyder et al., 1971; Paltauf, 1972].

**Figure 2. Schematic illustration of plasmalogen biosynthesis [Wanders et al., 1996a].**

**V. Amino acids and glyoxylate metabolism**

Peroxisomes contribute to gluconeogenesis by catabolism of amino acids. Basic and neutral D-amino acids are oxidatively deaminated to NH₃, H₂O₂ and their corresponding α-keto acids by D-amino acid oxidase [de Duve and Baudhuin, 1966]. The acidic D-amino acids, D-aspartate and D-glutamate, are oxidatively deaminated by D-aspartate oxidase [Zaar et al., 1989; van Veldhoven et al., 1991]. Furthermore, peroxisomes are involved in L-lysine metabolism, which can occur via saccharopine, or via L-pipecolic acid to α-amino adipate. The first route is most active in rat liver and other tissues but the latter is of major importance in the brain, as shown in the rat [Chang, 1978], human and monkey [Chang, 1982]. L-Pipecolic acid is oxidized by L-pipecolate oxidase to Δ¹-piperideine-6-carboxylate. L-pipecolate oxidase has been localized in peroxisomes of human liver [Wanders et al., 1989] and monkey liver [Mihalik et al., 1991] whereas, in rat brain and liver it is found both in peroxisomes as well as in
mitochondria [Rao et al., 1993]. In the Zellweger syndrome [Wanders et al., 1989], L-pipeolic acid accumulates due to a deficiency of this enzyme. Glutaryl-CoA, a catabolite of L-lysine, L-hydroxyproline and L-tryptophan has been reported to be oxidized by a presumed separate peroxisomal glutaryl-CoA oxidase [Bennett et al., 1991]. Whether there is a separate enzyme or not remains obscure [Van Veldhoven et al., 1992].

Aminotransferases play a role in removal of the α-amino group during catabolism of amino acids. Glyoxylate, formed from glycolate by glycolate oxidase [Yanagawa et al., 1990], is an intermediate precursor of oxalate (figure 3). Normally, glyoxylate and L-alanine are transaminated by L-alanine:glyoxylate aminotransferase I (AGT I) to glycine and pyruvate. In the human disease Hyperoxaluria type I, AGT I activity is strongly reduced [Danpure and Jennings, 1986], metabolism of glyoxylate is disturbed since transamination is blocked which leads to accumulation of glyoxylate and subsequent production of oxalate resulting in the formation of insoluble calcium-oxalate crystals in different organs (nephrocalcinosis, urolithiasis) in these patients. The subcellular distribution of AGT I is species specific. AGT I is exclusively peroxisomal in the human, monkey, rabbit and guinea-pig, mitochondrial in the dog and cat and localized in both organelles in the rat, mouse and hamster [Noguchi, 1987]. The human enzyme is peroxisomal [Noguchi and Takada, 1979] but in some Hyperoxaluria type I patients, AGT I is mistargeted to mitochondria [Danpure et al., 1989]. Here, peroxisome to mitochondrion mistargeting of the homodimeric enzyme is associated with the combined presence of a normally occurring Pro(11)Leu polymorphism and a PH1-specific Gly 170 Arg mutation, which impairs dimerization of AGT I therefore allowing mitochondrial import of the enzyme [Leiper et al., 1996].

Figure 3. Glycolate metabolism.

VI Polyamine and purine catabolism

Polyamine oxidase, identified in peroxisomes of rat liver [Hölttä, 1977a], kidney and duodenum [Van den Munckhof et al., 1995] oxidizes N1-acetylspermine and N1-acetylsperrmidine [Hayashi et al., 1989; Van den Munckhof et al.,
Polyamines are naturally occurring polycations involved in the regulation of differentiation and normal cell growth. Human purine catabolism ends up in uric acid, whereas most mammals degrade uric acid to allantoin by the action of urate oxidase. Urate oxidase constitutes the crystalline core of peroxisomes in liver.

VII Cholesterol and dolichol synthesis

The pathways for the synthesis of dolichol, ubiquinone, haem A, farnesylated proteins (Ras, Lamin B) and cholesterol are the same up to the level of farnesyl-pyrophosphate [Krisans, 1996; Reese and Maltese, 1991] (figure 4). The enzymes responsible for the synthesis of cholesterol [Thompson et al., 1987; Appelkvist, 1987a] and dolichol [Appelkvist, 1987b; Appelkvist and Kalen, 1989] from farnesyl-pyrophosphate have been identified in the ER (membrane associated) and have also been found in peroxisomes [Appelkvist et al., 1990; Ericsson et al., 1993]. There is some ambiguity with regard to the subcellular localization of squalene synthase, which according to some authors is exclusively localized to the ER [Cohen et al., 1992; Stamellos et al., 1993] whereas according to others the enzyme is present in ER and peroxisomes [Ericsson et al., 1993]. The important role of peroxisomes in cholesterol synthesis is also clear from studies by Mandel and coworkers [Mandel et al., 1995] who demonstrated a deficient de novo cholesterol synthesis in peroxisome deficient fibroblasts (see also [Hodge et al., 1991; Malle et al., 1995]). The initial part of the de novo synthesis pathway of isoprenoids is the two step conversion of acetyl-CoA to HMG-CoA. First, two molecules of acetyl-CoA combine to acetoacetyl-CoA by the catalytic action of acetoacetyl-CoA synthetase demonstrated to be present in mitochondria, cytosol and peroxisomes [Krisans, 1992; Hovik et al., 1991]. HMG-CoA synthase catalyses the addition of another acetyl-CoA unit to the acetoacetyl-CoA to form HMG-CoA. HMG-CoA reductase, the key regulatory enzyme in isoprenoid biosynthesis, catalyses the formation of mevalonate and is found to be present in the ER membrane as well as within the peroxisomal matrix [Keller et al., 1985]. No information is available regarding the function of the peroxisomal HMG-CoA reductase. Mevalonate kinase, which catalyses the formation of mevalonate-5-phosphate from mevalonate, is localized in rat liver peroxisomes [Stamellos et al., 1992]. Furthermore, this enzyme is deficient in liver from Zellweger patients [Wanders and Romeijn, 1996a]. The conversion of mevalonate-5-phosphate to farnesyl diphosphate (FPP) requires the following enzymes: mevalonate phosphate kinase, mevalonate diphosphate decarboxylase, isopentenyl diphosphate: dimethyl-allyl diphosphate (IPP) isomerase and farnesyl diphosphate (FPP) synthase (for review see [Krisans, 1996]). The peroxisomal enzymes involved in the conversion of mevalonate to dimethyl-allyl diphosphate have now been cloned and sequenced and shown to contain a conserved putative PTS1 or PTS2 which supports a targeted transport into peroxisomes [Chambliss et al., 1996; Toth and Huwyler, 1996; Paton et al., 1997]. FPP is then converted to cholesterol (figure 4). The last step in the conversion of 7-dehydrocholesterol to cholesterol has been known to be deficient in SLO (Smith Lemli Opitz syndrome) patients, since 7-dehydrocholesterol accumulates in these patients.
1.2.2 The biogenesis of peroxisomes

Since peroxisomes do not contain DNA, their membrane and matrix proteins have to be encoded by nuclear genes. The proteins are generally synthesized on free polyribosomes and posttranslationally imported into peroxisomes at their mature size, without a cleavable amino-terminal signal (for review see [Borst, 1989; Subramani, 1998]). Exceptions to this rule are: 3-ketoacyl-CoA thiolase [Furuta et al., 1982, Fujiki et al., 1985; Osumi et al., 1991; Swinkels et al., 1991], acyl-CoA oxidase [Osumi et al., 1980; Furuta et al., 1980; Miura et al., 1984], a non-specific lipid transfer protein [Fujiki et al., 1989] now known as SCP2 [Seedorf et al., 1994], which are synthesized in a precursor form (for review see [Elgersma and Tabak, 1996]). The current idea on peroxisome biogenesis which suggest that they simply arise from preexisting peroxisomes by budding and fission, has recently been put to discussion (for review see [Subramani, 1998]).

1.2.3 Protein targeting and import machinery

How does the nuclear encoded protein knows where to go? In recent years, it has become clear that peroxisomal proteins harbour a specific topogenic signal sequence within the polypeptide which allows proper sorting, together
with their cognate receptors, to the peroxisome-specific import machinery (figure 5).

**Figure 5. Model for import of peroxisomal matrix proteins.**

Proteins with COOH-terminal PTS1 and NH2-terminal PTS2 sequences are recognized by their cognate PTS receptors, Pex5p and Pex7p, respectively, in the cytosol. These PTS receptors bound to cargo then interact with docking proteins in peroxisomal membrane. Pex5p interacts with Pex7p, Pex13p. Pex7p interacts with Pex5p and Pex14p. In *S. cerevisiae* Pex7 is also found in the peroxisome (as shown in the picture), where it might act to facilitate import of PTS2-containing proteins [Zhang and Lazarow, 1995]. Protein unfolding is not required for import of matrix proteins into peroxisomes. This is depicted by transport of mixed oligomers (dimers and trimers), either containing or lacking PTS1 or PTS2 sequences. Derived from Subramani [1998].

Until now, several so called peroxisomal targeting signals (PTSs) have been identified which are classified as signals that target proteins to the peroxisomal matrix (PTS1, PTS2 and internal PTSs) or to the peroxisomal membrane (mPTS) (figure 6). The PTS1-signal consists of a simple carboxyl-terminal tripeptide with a consensus sequence of S(A,C)-K(R,H)-L(M) (figure 6) [Gould et al., 1989; Swinkels et al., 1992]. However, recent data suggest a much higher degree of degeneracy of the PTS1-signal [Elgersma et al., 1996a]. The PTS1 tripeptide is not cleaved from peroxisomal proteins, it cannot function at internal locations within a protein's primary structure and furthermore a free carboxy-terminus is a necessary component of the motif [Subramani, 1998]. The PTS2-signal has been identified thus far in a few peroxisomal proteins (for review see Subramani, 1998). In peroxisomal 3-ketothiolase, PTS2 consists of a NH2-terminal or internal nonapeptide with a sequence of XX-R(K)-L(V,I)-Q-XXXXX-H(Q)-L(A) [Osumi et al., 1991,
Swinkels et al., 1992]. Upon import, the PTS2 which is part of a large aminoterminal presequence is often removed by a peroxisomal protease [Subramani, 1998].

![Diagram of Peroxisomal Targeting Signals](image)

**MEMBRANE PROTEINS**

- mPTS
  - Cytosol
  - Matrix
  - EG. C. boidinii PMP47
  - 20 AA mPTS
  - EG. P. pastoris Pex3p

**MATRIX PROTEINS**

- PTS1 - A C-terminal Tripeptide
  - H2N RKL COOH
  - ARM COOH
  - CH
  - EG. luciferase

- PTS2 - An N-terminal or Internal Nonapeptide
  - H2N XXRLQXsHJTI COOH
  - TH
  - QA
  - EG. thiolase

### Figure 6. Peroxisomal targeting signals.

Peroxisomal targeting signals (PTSs) used for import of proteins into peroxisomal matrix (PTS1 and PTS2) and those (mPTSs) used for sorting to peroxisomal membrane are shown. Proteins in which these PTSs were first described are also shown. Functional variants of consensus PTS1 (for mammalian cells) and PTS2 (for yeast) are shown below sequence of amino acids (in 1-letter code) comprising each PTS. Derived from Subramani [1998].

mPTSs have been described in only two proteins, PMP47 in C. boidinii [Dyer et al., 1996] and Pex3p in P. pastoris [Wiemer et al., 1996] (figure 6). The PMP47 consists of six transmembrane segments in which the mPTS is a 20 amino acid loop, facing the matrix and located between the putative transmembrane domain 4-5, whereas Pex3p harbours the signal in the NH2-terminal 40-amino acid segment. The only homology observed is a charged stretch of 5 basic amino acids, whether these mPTSs are similar or distinct has to be resolved. Furthermore, upon import of the mPTS, it has been suggested that two pathways exist, one that directly targets the proteins from cytosol to peroxisome (mPTS1) whereas the other makes a "detour" via the ER (mPTS2). The exact mechanism remains to be elucidated. Recently, specific protein receptor molecules were discovered which are involved in peroxisomal targeting signal recognition (figure 5). Pex5p, encoded by the PEX5 gene, recognizes and binds PTS1 containing proteins. Pex5p is a member of the TPR protein family, which is characterized by the presence of multiple repeats of a degenerate consensus sequence [Goebl and Yanagida, 1991]. The other receptor molecule, Pex7p, has been suggested to function as a mobile receptor shuttling PTS2 containing proteins from the cytosol into the peroxisomes [Elgersma et al., 1998]. Pex7p is a member of the WD-40 (ß-transducin) protein family, which is characterized by the presence of a 43-amino acid repeat domain [Marzioch et al., 1994; Zhang and Lazarow, 1995]. Since TPR repeats and WD repeats are probably involved in multiple protein-protein interactions [Komachi et al., 1994] it has been suggested that the PTS1- and PTS2 receptors form a functional complex which is necessary for proper import of the PTS containing proteins [Dodt et al., 1995] (figure 5). So two distinct import pathways for peroxisomal matrix proteins exist, which turned out to be conserved from yeast to man [Subramani, 1998]. Upon import two peroxisomal membrane proteins, docking proteins, have been described that are essential for both PTS-dependent import pathways. Pex13p, an integral peroxisomal membrane protein, contains a SH3 domain which is required for docking.
of only the PTS1 receptor Pex5p [Elgersma et al., 1996a, Subramani, 1998]. Pex14p, a peripheral membrane protein located at the outer face of the peroxisome, interacts with various Pex proteins, i.e. Pex5p, Pex7p and the SH3 domain of Pex13p [Albertini et al., 1997]. Recently, Pex17p has been suggested to be involved in the peroxisomal import machinery [Hushe et al., 1998]. Whether or not other proteins encoded by PEX genes are involved in the sorting and translocation machinery, next to Pex5p, Pex7p, Pex13p, Pex14p and Pex17p, remains to be established.

Unfolding of protein prior to import, as is the case in mitochondrial protein import, appears to be unnecessary for peroxisomal protein import [Mathiew et al., 1994; Glover et al., 1994] or necessary as observed for AGT1 [Leiper et al., 1996]. Up to now 17 peroxins have been implicated in peroxisome biogenesis. Pex2p, Pex10p and Pex12p behave as integral membrane proteins containing Zn fingers. Pex1p and Pex6p are ATPases of the AAA family, these peroxins have been recently shown to be associated not only with peroxisomes but also with vesicles distinct from peroxisomes [Faber et al., 1998]. Pex4p is a 21-24 kDa peroxisome-associated ubiquitin conjugating enzyme. Pex8p plays a role in biogenesis harbouring PTS1 and PTS2 sequences and is present in the matrix of peroxisomes. Pex11p is a 27-32 kDa peroxisome associated protein involved in peroxisome proliferation. Pex16p and Pex17p are directly or indirectly involved in matrix protein import.

For more information about peroxisome import, biogenesis, proliferation the reader is referred to the reviews on this subject [Elgersma and Tabak, 1996; Subramani, 1998]. For unified protein and gene nomenclature of peroxisome biogenesis factors see [Distel et al., 1996].

1.2.4 The ability of peroxisomes to proliferate and its mechanism

A unique feature of peroxisomes is their massive protein induction and organelle proliferation as observed in yeast and rodents upon several feeding conditions. Methanol, long-chain fatty acids, fibrate hypolipidemic drugs, phthalate ester plasticizers and trichloroacetic acid herbicides can induce peroxisome proliferation and are thus collectively called peroxisome proliferators (PPs). Insight into their mechanism of action was provided by the identification of a member of the nuclear hormone receptor (NHR) superfamily activated by PPs [Isseman and Green, 1990]. Characteristic of this NHR family is that these proteins harbour a central DNA binding domain (DBD) which targets the receptor to cis-acting hormone response elements (HREs) in the promoter of target genes. Once bound to a HRE, each receptor responds to its specific ligand through C-terminal ligand binding domain (LBD) which binds its cognate hormone with high affinity and specificity. Indeed, peroxisome proliferator activated receptor (PPAR) was identified and shown to be activated by a variety of fatty acids and subsequently stimulated the expression of the genes encoding β-oxidation enzymes which concomitantly increased the capacity of peroxisomes to metabolize fatty acids (for review see [Reddy and Manneerts, 1994]). PPARs belong to the HNR subfamily which comprise the thyroid hormone (TR), retinoic acid (RAR), vitamin D and ecdysone receptors and the orphan receptors EAR-1 and E75. This class of receptors preferentially bind to DNA as heterodimers with a common partner, the 9-cis-retinoic acid receptor (RXR). Indeed, PPAR-RXR complex have been identified and shown to bind to peroxisome responsive
elements (PPRE) present in peroxisomal genes thereby enhancing their rate of transcription. PPRE consists of direct repeats with a consensus sequence of TGACCT separated by one base pair (DR1) [Issuman and Green, 1990] (figure 7). Several receptors, PPARα, PPARβ, PPARγ, have been identified and revealed a less conserved LBD in these receptors, indicating that each receptor possesses a differential response profile. Moreover, their tissue expression is different. PPARβ was found to be the most ubiquitously expressed subtype in rat, suggesting a general role. In contrast, PPARα is highly expressed in tissues like liver, heart and muscle, with high β-oxidation activities in mitochondria and peroxisomes. Genes encoding for key enzymes involved in β-oxidation and ketogenesis are regulated by PPARα. This process is induced upon fasting and stress. Instead, PPARγ expression is only seen in part of the immune system and in white and brown adipose tissue. Recently, PPARγ has been described to play a key role in adipocyte differentiation and furthermore promotes storage of lipids (adipogenesis). PPARα has been shown to bind and to be activated by leukotriene B4 and fibrates whereas prostaglandin J2 derivatives and the thiazolidinediones are the natural and synthetic ligands, respectively, for PPARγ [Devchand et al., 1996]. However their mechanism of action has not yet been fully defined (for recent review see [Latruffe and Vamecq, 1997]).

It has become clear that despite their name PPARs also influence the regulation of mitochondrial fatty acid metabolism. They influence not only activation through the control of acyl-CoA synthetase [Schoonjans et al., 1995], β-oxidation, through medium-chain acyl-CoA dehydrogenase [Gulich et al., 1994] but also mitochondrial import through CPT I [Mascaró et al., 1998].

**Figure 7. Proposed model of peroxisomal proliferator action [Tugwood et al., 1996].** (a) Glucocorticoid receptor (GR), TR, estrogen receptor (ER), RXR and PPAR as parts of a hormone-signalling network. The interaction between the PPAR signalling pathway and that of other hormone signalling pathways as described by Lemberger et al. [1996] (b).

### 1.3 Mitochondria

Compartmentation is not restricted to cells only, even within one organelle compartmentation can occur. The mitochondrion for instance contains four compartments where enzymes may be localized: 1. the outer membrane; 2.
the inter membrane space, i.e. the space between the outer and inner membranes, including the space bordered by the outer surface of the cristae; 3. the inner membrane, including the cristae and their projecting subunits, and 4. the matrix, i.e. the space within the inner surface of the inner membrane (for review see [Ernster and Kuylenstierna, 1969]). In liver mitochondria the two membranes are approximately equal in thickness 50-70 Å [Parsons, 1965] but differ in their surface area, the inner membrane is greatly enlarged owing to cristae. The outer and inner membranes differ in:

a) permeability;

The outer membrane is permeable to a wide range of charged and uncharged compounds up to 10 kDa whereas the inner membrane has a limited permeability to uncharged molecules and the majority of the charged molecules pass by way of specific translocators embedded in the membrane.

b) Lipid composition;

The outer membrane contains, on protein basis, twice as much total phospholipid as compared with the inner membrane. Phosphatidyl-inositol is more abundant in the outer membrane whereas cardiolipin occurs predominantly in the inner membrane. Cholesterol is more abundant in the outer membrane and ubiquinone is present only in the inner membrane (for review see [Ernster and Kuylenstierna, 1969]). Mitochondria harbour enzymes involved in a broad spectrum of metabolic functions i.e. synthesis of amino acids, haem, pyrimidines, sugars, lipids, β-oxidation of fatty acids and respiration-driven synthesis of ATP. The respiratory chain, located in the inner mitochondrial membrane, consists of four different enzyme complexes (complex I, II, III, IV) which transfer electrons from NADH or succinate to molecular oxygen. This leads to generation of a proton gradient across the inner mitochondrial membrane which is used by the F_{i}F_{o}-ATP-synthase complex (complex V) for ATP synthesis. In most eukaryotic cells production of ATP is mainly via the oxidative phosphorylation system.

1.3.1 The biogenesis of mitochondria

Mitochondria arise by growth and division of pre-existing mitochondria. This growth occurs by insertion of newly synthesized constituents, which results in expansion of the various compartments of each mitochondrion [Neupert, 1997]. The biogenesis of mitochondria represents a complex process, which involves controlled synthesis of proteins and some phospholipids inside the pre-existing organelle and a concomitant synthesis of proteins in the ER, followed by transfer to the intact mitochondrion. Mitochondria like chloroplasts contain DNA (mtDNA) encoding proteins which are synthesized on mitochondrial ribosomes and inserted from the matrix side into the inner membrane. In mammals these mtDNA-encoded proteins are hydrophobic subunits of membrane enzymes all functioning in oxidative phosphorylation [Attardi and Schatz, 1988].

The oxidative phosphorylation system consists of oligomeric complexes which are in part encoded by mtDNA and in part by nuclear DNA (nDNA). Human mtDNA has been fully sequenced [Anderson et al., 1981] and has been shown to code for 2 rRNA's, 22 tRNA's and 13 subunits of the oxidative phosphorylation system, i.e. 7 subunits of complex I, 1 subunit (apo-cytochrome b) of complex III, 3 subunits of complex IV, 2 subunits of complex V (F_{i}F_{o}-ATPase). All
other mitochondrial proteins are encoded by nuclear genes, synthesized as precursors in the cytoplasm and imported into one of the four mitochondrial compartments: outer membrane, inter membrane space, inner membrane, and matrix. The replication and transcription of mtDNA and the translation of mtDNA-coded mRNA's require components encoded in the nucleus, which must be imported into the organelle. The biogenesis of mitochondria therefore depends on the correct expression of both mitochondrial and nuclear genes [Attardi and Schatz, 1988]. Impairment in expression of mtDNA genes or nDNA genes involved in mitochondrial biogenesis can result in mitochondrial dysfunction [Holt et al., 1988].

1.3.2 Protein targeting to the mitochondria

In mammalian cells, nuclear coded mitochondrial precursor proteins with cleavable amino-terminal extensions interact with cytosolic unfolding proteins such as heat shock proteins (Hsp 70), pre-sequence binding factors (PBF of 50 kDa) or mitochondrial import stimulation factors (MSF, a heterodimer of 30 and 32 kDa subunits), which prevent tight folding of the precursor protein before mitochondrial trans-membrane passage (for review see [Neupert, 1997]). MSF is thought to promote both depolymerization of the oligomeric precursor and unfolding in an ATP dependent manner. Mitochondrial targeting sequences are generally rich in positively charged and hydroxylated amino acids and have the capability of forming amphiphilic α-helices (or possibly β-sheets) when in contact with lipid bilayer, a characteristic which is thought to be crucial to their function in mitochondrial import (for review see [Neupert, 1997]). At the mitochondrial outer membrane, import of preproteins is initiated through a series of reactions, which include preprotein recognition, unfolding, insertion and translocation. These processes are facilitated by a multi subunit complex, recently called the TOM complex (for Translocase of the Outer Membrane of mitochondria) (for uniform nomenclature see [Pfanner et al., 1996]). Specific roles can now be assigned to several components of this complex. Preproteins, with a preference for preproteins that have an amino-terminal presequence or internal targeting information, are recognized by import receptors (TOM 70, TOM 71, TOM 37 and/or TOM 22, TOM 20) on the mitochondrial surface. Translocation across the outer membrane occurs by further translocase components including, TOM 40 an essential constituent of the general import pore, TOM 7 and TOM 6 which modulate the assembly and dissociation of the TOM machinery and TOM 5 which plays a crucial role in importing preproteins destined for all four mitochondrial subcompartments (fig 8). The TOM proteins can assemble into a loose complex (for review see [Neupert, 1997]). The recent isolation of a human homologue of a TOM complex protein suggests, that the protein import machinery is conserved throughout the eukaryotic kingdom [Goping et al., 1995]. Although the import machinery of the outer membrane can insert and translocate a few proteins on its own, completion of translocation of most preproteins is dependent upon coupling to both the membrane and mtHsp 70/ATP-driven transport across the inner membrane, mediated by the TIM complex (for Translocase of the Inner Membrane of mitochondria) (for uniform nomenclature see [Pfanner et al., 1996]). TIM 17 and TIM 23 are thought to build a preprotein translocation channel, while TIM 44 transiently interacts with the matrix heatshock protein Hsp70 to form an ATP-driven import motor (fig 8). Recently, it has been suggested that the inner mitochondrial membrane
harbours an additional TIM import complex (TIM 54p-TIM22p) which is required for the insertion of proteins into the inner membrane [Kerscher et al., 1997]. In this process import of multspanning carriers is facilitated by TIM 10p and TIM 12p present in the intermembrane space [Koehler et al., 1998].

Figure 8. Schematic representation of components of the TOM and TIM complex.
Abbreviations: OM, outer membrane; IMS, intermembrane space; IM, inner membrane. (adapted from [Neupert, 1997]).

Once inside the mitochondrial matrix, precursor proteins can be proteolytically cleaved (removing their matrix targeting sequence) by matrix processing peptidase (MPP). MPP consists of two components, α-MPP and β-MPP, which are both required for activity. In plants, MPP has been shown to be an integral part of the cytochrome bc1 or cytochrome c reductase complex. Whether this is the case in humans remains to be established [Neupert, 1997]. It is not an essential feature for mitochondrial proteins that they possess a cleavable pre-sequence, mitochondrial 3-oxo-acyl-CoA thiolase for instance lacks a cleavable presequence (for review see [Neupert, 1997]). It was proposed that the sequence requirements for mitochondrial import and recognition of MPP are distinct. The mitochondrial intermediate peptidase (MIP) is responsible for the removal of octapeptides remaining on some precursors after their cleavage by MPP in the matrix. During or after the process of entering the matrix space, preproteins are folded into their native state, a process in which molecular chaperones play an important role (see table 5 in [Neupert, 1997]).

The above described import pathway is the general pathway that leads preproteins into the matrix of mitochondria. At least three pathways exist in the case of targeting proteins to the intermembrane space (IMS):

1) Cytochrome c haem lyase (CCHL) is a protein synthesized without a cleavable pre-sequence and is the only known TOM complex dependent protein which is imported directly across the OM (via the translocation pore) without requirement for ATP or a membrane potential.

2) Cytochrome c, a soluble component of the intermembrane space without a cleavable targeting sequence, takes a unique route. It traverses the outer membrane, without involvement of the TOM complex by penetrating the lipid bilayer and then becomes trapped inside the IMS by binding to CCHL.
3) The targeting mechanism to the IMS of precursors, such as cytochrome b$_2$ and c$_1$, that contain bipartite signal sequences remains a controversial issue. The most N-terminal signal of these precursors is a matrix-targeting sequence, followed by a second signal termed the sorting signal. There is disagreement about the manner in which this sorting signal operates in targeting precursors to the IMS; a) it may function as a stop-transfer domain that arrests the imported protein in the IM or b) it may function as a conservative sorting domain in which proteins are imported along the general import pathway into the matrix from where they embark on the export pathway across the IM to the IMS.

The process of sorting proteins to and integration into the inner mitochondrial membrane can be grouped according to the structure of the precursor proteins i.e. with or without a cleavable N-terminal signal. In case of a cleavable N-terminal target sequence, present in subunit 9 of F$_1$F$_0$-ATPase and subunit II of cytochrome oxidase (COX II), the target signal is removed in the matrix whereafter the N-terminal transmembrane segment is inserted into the inner membrane in a ΔμH$^+$ dependent process. The targeting and insertion of proteins without a cleavable N-terminal targeting sequence, as is the case with the ADP/ATP translocator and related carrier proteins, which may contain more than one internal signal in their mature sequence, still remains to be elucidated (for reviews see [Lill et al., 1996; Neupert, 1997; Pfanner and Meijer, 1997]).

1.4 Fatty acid metabolism

Fatty acids play a major role as integral components of membrane lipids and as highly concentrated energy stores, in the form of triacylglycerols. Fatty acids are derived either from blood plasma, where they are bound non-covalently to serum albumin or esterified in triacylglycerols of lipoproteins and chylomicrons, or derived from intracellular triacylglycerols. Long-chain fatty acids constitute the bulk of fatty acids present in the organism and therefore are a major source of metabolic fuel. This is particularly apparent in heart tissue where fatty acids are utilized in preference to glucose for the production of energy.

1.4.1 The mobilisation of fatty acids

Several lipases which can hydrolyse triacylglycerols into free fatty acids have been identified: lipoprotein lipases acting at the endothelial surface of the vascular compartment, hormone-sensitive lipase (HSL) acting in adipose tissue, heart and skeletal muscle and lysosomal triacylglycerol lipase acting intracellularly in the liver (for review see [Guzmán and Geelen, 1993]). Free fatty acids mainly arise from lipolysis of triacylglycerols by the action of HSL in adipose tissue. HSL activity is induced by noradrenaline, epinephrine, glucagon, adrenocorticotropic hormone (ACTH) and thyroid hormone (see figure 7 for hormone signalling pathway). The plasma concentration of free fatty acids varies from 0.2 to 0.5 mM but can rise to 1.5 mM and higher during prolonged fasting, exercise or disease. Fatty acids in plasma and cells are mainly bound by membranes and proteins such as albumin and fatty acid binding proteins (FABP), which can regulate their biological activities and metabolic transformations. The rate of entry of fatty acids into cells depends on their plasma concentrations and metabolic activity of the tissue. It is assumed that
long-chain fatty acids enter the cell by one of 2 mechanisms: at low concentrations, via a saturable carrier mechanism and at high concentrations, by direct diffusion across the plasma membrane (for review see [Guzmán and Geelen, 1993]). Several plasma membrane proteins having ability to bind LCFAs or their analogues have been proposed as putative membrane transporters of LCFAs, among which fatty acid transport protein (FATP) and fatty acid translocase (FAT) have so far been analyzed by gene cloning and their biochemical studies [Schaffer and Lodish, 1994; Abumrad et al., 1993]. FATP, identified in 3T3-L1 adipocytes, is a 63 kDa integral plasma membrane protein with six transmembrane spanning regions [Schaffer and Lodish, 1994]. FAT is a 88 kDa plasma membrane glycoprotein with two predicted transmembrane domains [Abumrad et al., 1993]. Close investigation revealed that expression of the FATP gene is down-regulated by insulin and up-regulated upon fasting [Man et al., 1996]. Recently, it has been suggested upon differential regulation by cytokines of FATP(-) and FAT (+) mRNA in liver, that these proteins may be involved in transporting fatty acids to different locations inside the cell. FATP may be involved in transporting FAs for mitochondrial oxidation whereas FAT may transport FAs towards cytosol for re-esterification [Memon et al., 1998].

Once inside the cell, LCFAs probably bind to FABPs which are found in the cytosol of many tissues. The FABP gene family consists of structurally related cytosolic 14-15 kDa proteins which have in common, the binding of low molecular weight hydrophobic and amphiphatic ligands (for review see [Coe and Bernlohr, 1998]). FABPs are assumed to play a specific role in intracellular transport and metabolism of fatty acids. Fatty acids bound to FABPs are transported to ER, mitochondria or peroxisomes depending on the metabolic demand of the cell, i.e. formation of acylglycerols, fatty acid synthesis or oxidation. In addition to the various FABPs, an acyl-CoA binding protein (ACBP) has been identified that is localized in the cytosolic compartment [Vanden heuvel et al., 1993]. It has been suggested that ACBP is able to act as an intracellular acyl-CoA transporter and pool former, although the exact function of this protein is unclear at present.

1.4.2 Activation of fatty acids to their corresponding CoA ester

The initial and obligatory step in fatty acid metabolism is their activation to their corresponding CoA esters by synthetases (ligases) at the expense of 1 molecule of ATP yielding AMP plus pyrophosphate (for review see [Groot et al., 1976]). Several synthetases exist which catalyze the same type of reaction but differ in their subcellular localization and utilization of (different chain-length) fatty acids.

Short-chain acyl-CoA synthetases use acetate, propionate or butyrate as substrates and are found in the mitochondrial matrix and/or cytosol, depending on the tissue (for review see [Groot et al., 1976]). Scholte and Groot [1975] suggested that the mitochondrial enzyme is involved in oxidation whereas the cytosolic enzyme is involved in fatty acid synthesis. Medium-chain acyl-CoA synthetases act on fatty acids with a chain-length of 4-12 carbons and are located in the mitochondrial matrix [Aas, 1971; Killenberg et al., 1971]. Long-chain acyl-CoA synthetases catalyze the activation of saturated- (C10-C18) and unsaturated (C16-C20) fatty acids [Tanaka et al., 1979; Christensen et al., 1993] but also the branched chain fatty acid pristanic acid [Wanders et al., 1992a]. These
enzymes are membrane bound and associated with the outer mitochondrial membrane, the ER membrane and the peroxisomal membrane facing the cytosol [Mannaerts et al., 1982; Miyazawa et al., 1985, Lageweg et al., 1991; Lazo et al., 1988]. The different long-chain acyl-CoA synthetases as purified from the different cell fractions were found to be indistinguishable [Hashimoto, 1982; Miyazawa et al., 1985]. Suzuki and coworkers [Suzuki et al., 1990] cloned and sequenced the rat liver long-chain acyl-CoA synthetase cDNA, which was further studied by the group of Watkins using an in vitro transcription/translation system [Watkins et al., 1996]. It turned out that this long-chain acyl-CoA synthetase is capable of activating not only palmitic acid but also phytanic acid. On the other hand Singh and coworkers [Pahan et al., 1993] identified a new peroxisomal acyl-CoA synthetase in human skin fibroblasts, distinct from all the previously mentioned acyl-CoA synthetases, which activates phytanic acid to phytanoyl-CoA prior to its α-oxidation. Whether or not there is this distinct enzyme in human fibroblasts for activation of phytic acid to its CoA ester remains to be resolved. Until now the enzyme has not been purified. Dicarboxylyl-CoA synthetase and THCA-CoA synthetase catalyze the activation of dicarboxylic fatty acids and di- and trihydroxycholestanolic acids, respectively. These synthetases are membrane bound and localized in the ER [Vamecq et al., 1985; Schepers et al., 1989]. Whether or not leukotrienes [Jedlitschky et al., 1991] and prostaglandins [Schepers et al., 1988] are activated by separate synthetases remains to be elucidated. Very-long-chain acyl-CoA synthetases as their name indicates activate very-long-chain fatty acids to their corresponding CoA ester and are localized in the peroxisomal membrane and the ER [Wanders et al., 1987a; Singh H et al., 1987a,b] but not in mitochondria [Wanders et al., 1987a; Lazo et al., 1988]. There is contradiction about the catalytic site of the enzyme at the peroxisomal membrane, either facing the cytosol [Lageweg et al., 1991] or facing the peroxisomal interior [Lazo et al., 1990]. Recently, the peroxisomal very-long-chain acyl-CoA synthetase was purified and cloned [Uchida et al., 1996]. In patients affected by X-linked adrenoleukodystrophy (X-ALD) oxidation of very-long-chain fatty acids is deficient resulting in an accumulation of these fatty acids, especially in the brain. For a long time it was thought that X-ALD was caused by a deficiency of the peroxisomal very-long-chain acyl-CoA synthase. Since Mosser and coworkers [Mosser et al., 1993] identified the gene involved in X-ALD and found that it encodes a protein belonging to the superfamily of A(TP) B(inding) C(assette)-transporters, it has become clear that X-ALD is a peroxisomal disease in which the defect is due to a genetic defect in a transport protein. Other members of the ATP family are the cystic fibrosis transmembrane conductance regulator (CFTR), the multidrug resistance protein (MDR) and also the peroxisomal 70 kDa membrane protein (PMP70).

1.4.3 Transport of the fatty acids into the organelles
Prior to their oxidation, fatty acids have to be transported into the organelles, i.e. peroxisomes or mitochondria. Medium- and short-chain fatty acids which are predominantly oxidized in mitochondria [Mannaerts et al., 1982], are transported as such across the mitochondrial membrane and activated to their corresponding CoA-ester in the mitochondrial matrix. On the other hand, mitochondrial long-chain fatty acid β-oxidation is dependent on carnitine (Fig 9 A) [McGarry et al., 1992]. Long-chain fatty acyl-CoAs, formed at the mitochondrial outer membrane, cannot
cross the inner membrane due to impermeability of the inner membrane towards CoA-esters. At the outer membrane, long-chain fatty acyl-CoAs are converted to long-chain acyl-carnitines by carnitine-palmitoyltransferase I (CPT I) [Murthy and Pande, 1987]. Via carnitine/acylcarnitine translocase, present in the inner membrane, acyl-carnitines are transported through the inner membrane in exchange for mitochondrial carnitine [Pande, 1975; Indiveri et al., 1991]. Carnitine-palmitoyltransferase II (CPT II), at the mitochondrial inner membrane facing the matrix, converts long-chain acyl-carnitine to long-chain acyl-CoA after which β-oxidation can occur [Bieber, 1988; McGarry et al., 1992]. Two isoforms of CPT I have been described which are mainly expressed in liver (ICPT I) and muscle (mCPT I), although expression of the mCPT I gene is not only in skeletal muscle but also in heart and brown and white adipose tissue [Weis et al., 1994; Yamazaki et al., 1995, 1996; Esser et al., 1996]. Mascaro and coworkers recently showed that mCPT I gene expression is regulated by fatty acids and peroxisome proliferators at the transcriptional level by PPARs [Mascaro et al., 1998]. A functional characterization of CPT I revealed a single CPT I polypeptide harbouring the CPT I catalytic activity just as the malonyl-CoA sensitivity, in mammalian mitochondrial membranes [de Vries et al., 1997]. In contrast to CPT I, CPT II is insensitive to malonyl-CoA and detergent stable [Bieber, 1988; McGarry et al., 1989]. The cDNA for CPT II has been cloned both from human [Finocchiaro et al., 1991] and rat liver [Woeltje et al., 1990b]. CPT II is first synthesized as a precursor protein of 74 kDa, followed by processing to the mature membrane inserted enzyme of 71 kDa. The carnitine/acylcarnitine translocase, intensively studied by the group of Palmieri, acts by a Ping Pong reaction mechanism [Indiveri et al., 1994]. This carrier protein, isolated from rat liver mitochondria as a 32.5 kDa protein [Indiveri and Palmieri, 1989], has recently been sequenced [Indiveri et al., 1997] and assigned to the mitochondrial carrier family.

Kerner and Bieber [1990] suggested that CPT I and CPT II are associated with a complex of β-oxidation enzymes. In vivo, this would allow substrate channelling from cytosol to mitochondrial matrix without diffusion. CPT I regulates the flux of long-chain fatty acids into the mitochondria for subsequent β-oxidation [Spurway et al., 1997]. The enzyme is inhibited by malonyl-CoA, which is synthesized by acetyl-CoA carboxylase and is the first intermediate of fatty acid synthesis [McGarry et al., 1977]. Enzyme activity is also modulated by insulin [Guzmán and Geelen, 1988], whereas glucagon renders the enzyme more active [Guzmán and Geelen, 1988]. During lipogenesis, in the fed state, when malonyl-CoA levels are high, CPT I is inhibited, arresting fatty acyl-CoA entrance into mitochondria and therefore making fatty acyl-CoAs accessible towards glycerolipid synthesis. During starvation, malonyl-CoA levels are low, CPT I is freely accessible for fatty acyl-CoAs, and fatty acyl-CoAs are directed towards mitochondrial β-oxidation.

In contrast to mitochondrial long-chain fatty acid oxidation, peroxisomal long-chain fatty acid oxidation (Fig 1 B) is thought to proceed independently of carnitine [Thomas et al., 1980; Appelkvist and Dallner, 1980], although Buechler and Lowenstein [1990] proposed an involvement of mitochondrial CPT I in supplying acyl-carnitines for peroxisomal β-oxidation. In chapter 5 and 6 we provide direct evidence for the involvement of carnitine in peroxisomal β-oxidation in such a way that chain-shortened fatty acids are shuttled from peroxisomes to
mitochondria as carnitine esters for further mitochondrial β-oxidation [Jakobs and Wanders, 1995]. No further evidence exists for the theory proposed by Buechler and Lowenstein.

Figure 9. Long-chain fatty acid transport across the mitochondrial and peroxisomal membranes (see text, for explanation).

How CoA esters synthesized in the ER or at the peroxisomal membrane enter the peroxisomal matrix for β-oxidation has not been fully understood. In the case of VLCFAs it has been speculated that they are transported either as such [Singh I et al., 1992; Lazo et al., 1990] or in the activated form [Lageweg et al., 1991a], depending on their site of activation.

At least four half ATP-binding cassette (ABC) transporters have been identified in the human peroxisomal membrane: the 70 kDa peroxisomal membrane protein (PMP70) [Kamijo K et al., 1992], the adrenoleukodystrophy (ALD) protein (ALDP) [Mosser et al., 1994], the ALDP-related protein [Lombard-Platet et al., 1996] and the 73 kDa transporter most similar to PMP70 designated P70R [Shani et al., 1997]. ATP-binding cassette (ABC) transporters are members of a superfamily of proteins involved in the transport of a variety of molecules ranging from ions to proteins across biological membranes [Higgins, 1992]. The first evidence of a peroxisomal ABC transporter involved in fatty acid metabolite transport was established by the group of Tabak [Hettema et al., 1996], using S. cerevisiae mutants, in which the gene coding for the yeast homologue of the human ALD-protein (Pxa2p) and peroxisomal ALDP-related protein (Pxa1p), was disrupted. These investigators showed that two independent pathways of peroxisomal fatty acid uptake exist: one for medium-chain fatty acids, which has been shown to be dependent on the peroxisomal acyl-CoA synthetase localized inside yeast peroxisomes, and the other for activated long-chain fatty acids, which is dependent on the peroxisomal ABC transporters Pxa1p and Pxa2p. Half ABC transporters contain a transmembrane domain and an ATP binding domain and are thought to dimerize, thereby forming a functional transporter. Indeed, it has been demonstrated that Pxa1p and Pxa2p heterodimerize to form a peroxisomal ABC transporter which is involved in β-oxidation of fatty acids at least in yeast [Shani and Valle, 1996].
investigators suggested that in mammalian cells various combinations of peroxisomal half ABC transporters assemble into functional transporters with different properties.

1.4.4 The β-oxidation cycle

Peroxisomal fatty acid β-oxidation occurs in animals, plants and unicellular eukaryotes whereas mitochondrial fatty acid β-oxidation only seems to occur in animal cells. Inside the organelle, fatty acid oxidation proceeds by a repeated sequence of flavoprotein linked dehydrogenation introducing a double bond, hydration, NAD+-linked dehydrogenation and thiolysis to generate acetyl-CoA (figure 10). The 2 carbon atoms shortened acyl-CoA ester can re-enter the β-oxidation spiral [Lazarow, 1978]. The acetyl-CoA released in β-oxidation can be oxidized in the Krebs-cycle, although in the liver acetyl-CoA can also be converted into ketone bodies.

Figure 10. Mitochondrial and peroxisomal β-oxidation.

Acetyl-CoA released in peroxisomal β-oxidation can be used for chain elongation [Horie et al., 1989], for biosynthesis of cholesterol [Krisans, 1992; Hovik et al., 1991] or can either be converted to acetate [Leighton et al., 1989] by an acetyl-CoA hydrolase [Alexson et al., 1989] or, to acetyl-L-carnitine by carnitine acetyltransferase(CAT) [Markwell et al., 1973]. Farrell and Bieber [1983] suggested that CAT and COT (carnitine octanoyltransferase) present in the peroxisomal matrix are involved in shuttling oxidation end-products from peroxisomes to mitochondria for further oxidation. Recently, we showed that carnitine is involved in peroxisomal β-oxidation, by shuttling peroxisomal β-oxidation products as carnitine esters to the mitochondria for further oxidation [Jakobs and Wanders, 1995; this thesis, chapter 5 and 6]. Since malonyl-CoA inhibits not only CPT I but also peroxisomal COT [Nic
A'Bhaird and Ramsay, 1992), it has been suggested that regulation of COT and CPT I in parallel may be necessary for the control of cellular fatty acid metabolism. Although the same set of reactions occurs in β-oxidation, the enzymes involved in peroxisomal and mitochondrial β-oxidation are encoded by different genes.

I Mitochondrial β-oxidation

There exist at least two enzymes with overlapping chain length specificity for each reaction step of mitochondrial β-oxidation. The initial step of β-oxidation is catalyzed by an FAD-dependent acyl-CoA dehydrogenase. Acyl-CoA is oxidized introducing a double bond to form a 2-trans-enoyl-CoA and FADH$_2$. In turn, FADH$_2$ donates its electrons to the ubiquinone pool of the mitochondrial respiratory chain by a specific system involving electron transfer flavoprotein (ETF) and electron transfer flavoprotein ubiquinone oxidoreductase (ETF-QO). Four different dehydrogenases have been identified for straight chain fatty acid oxidation with distinct specificity for very-long- (VLCAD), long- (LCAD), medium- (MCAD), and short-chain acyl-CoAs (SCAD) [Ikeda et al., 1985; Izai et al., 1992]. Furthermore, two types of dehydrogenases, short/branched chain acyl-CoA- (SBCAD) and isovaleryl-CoA dehydrogenase (IVD), have been identified which are involved in the oxidation of isoleucine/valine and leucine, respectively [Ikeda and Tanaka, 1983a,b]. The acyl-CoA dehydrogenases (ACDs) purified from rat [Ikeda, et al., 1985; Izai et al., 1992; Ikeda and Tanaka, 1983a] and human [Finnocchario et al., 1987] liver are homotetramers and located in the mitochondrial matrix, except VLCAD, a heterodimer, is localized in the inner membrane [Izai et al., 1992]. Mannaerts and coworkers [Vanhove et al., 1993a] showed that SCAD is capable, in the absence of suitable electron acceptors, to act as an oxidase by transferring electrons directly to molecular oxygen, yielding H$_2$O$_2$ (toxic). The ACDs show homology with the peroxisomal acyl-CoA oxidases [Baumgart et al., 1996b].

The second step in mitochondrial β-oxidation is catalyzed by 2-trans-enoyl-CoA hydratase which hydrates the trans double bond of 2-trans-enoyl-CoA to form L-3-hydroxyacyl-CoA. Two types of hydratases are known with broad overlapping substrate specificities, a short chain enoyl-CoA hydratase (crotonase) and long-chain enoyl-CoA hydratase (membrane bound) [Furuta et al., 1980]. Long-chain enoyl-CoA hydratase is part of the trifunctional protein which consists of two subunits, an α-subunit with enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activity and a smaller β-subunit with the thiolase activity. This protein has been shown to be present in rat and man bound to the inner mitochondrial membrane [Carpenter et al., 1992; Uchida et al., 1992] and shows activity towards acyl-CoAs with chain lengths of C8 up to C16 [Hashimoto, 1992]. This suggests that trifunctional protein plays a role in the first stage of the fatty acid oxidation spiral. The cDNA for both subunits of the trifunctional protein have been cloned [Kamijo T et al., 1994]. It has been suggested [Pollitt, 1995] that an additional medium chain enoyl-CoA hydratase exists, although the enzyme has not been purified.

The third step in mitochondrial β-oxidation is the dehydrogenation of L-3-hydroxyacyl-CoA to 3-ketoacyl-CoA (introducing a keto-group) by L-3-hydroxyacyl-CoA dehydrogenase. NAD$^+$ accepts the electrons in this reaction and donates them to complex I of the mitochondrial respiratory chain. It seems likely that this is a tightly linked process with its own specific NAD$^+$/NADH pool [Eaton et al., 1993]. Two mitochondrial L-3-hydroxyacyl-CoA dehydrogenases...
have been identified so far: the first one is a soluble L-3-hydroxyacyl-CoA dehydrogenase named short chain dehydrogenase but is active with C4 to C16 substrates whereas the other is part of the α-subunit of the membrane-bound trifunctional protein and active towards long-chain 3-hydroxyacyl-CoAs [Osumi and Hashimoto, 1979a; Osumi and Hashimoto, 1980; El-Fakhri and Middleton, 1982; Hashimoto, 1992].

Finally, 3-ketoacyl-CoA esters are cleaved at the β-position by 3-ketoacyl-CoA thiolas of which three types exist in mitochondria: 1) short-chain 3-ketoacyl-CoA thiolase involved in ketone-body production acting on acetoacetyl-CoA and 2-methylacetoacetyl-CoA [Middleton, 1973]; 2) “general” 3-ketoacyl-CoA thiolase which is active towards substrates of various chain lengths [Middleton, 1973]; 3) long-chain 3-ketoacyl-CoA thiolase located on the β-subunit of the membrane-bound trifunctional protein [Carpenter et al., 1992; Uchida et al., 1992].

The NADH and FADH$_2$ produced in mitochondrial β-oxidation are oxidized by the respiratory chain to produce ATP by oxidative phosphorylation.

II Peroxisomal β-oxidation

Peroxisomes oxidize a variety of fatty acids and their derivatives and for the first reaction at least two to three acyl-CoA oxidases have been characterized [for review see [Reddy and Mannaerts, 1994]). The initial step of β-oxidation is catalyzed by one of a variety of FAD-containing acyl-CoA oxidases, which directly react with molecular oxygen to yield H$_2$O$_2$ which is easily decomposed by catalase [Lazarow and de Duve, 1976] (fig. 10 and 11). Depending on tissue or species, several acyl-CoA oxidases exist. In rat liver peroxisomes three acyl-CoA oxidases have been shown to be present, 1) a clofibrate inducible palmitoyl-CoA oxidase, also present in extra-hepatic tissue, oxidizes the CoA-esters of medium-, long-, very-long-straight-chain fatty acids, medium- and long-chain dicarboxylic fatty acids and prostaglandins [Van Veldhoven et al., 1992]. This enzyme, purified from induced rat liver [Inestrosa et al., 1980; Osumi et al., 1980] has a molecular mass of 150 kDa and consists of three subunits of 72, 52 and 21 kDa, which are formed in vivo by posttranslational cleavage of the larger product [Osumi et al., 1980; Furuta et al., 1980; Miura et al., 1984]. 2) a non-inducible pristanoyl-CoA oxidase, also present in extra-hepatic tissue, oxidizes 2-methyl-branched-chain-, very-long-straight-chain- and long-chain dicarboxylic acyl-CoAs. This enzyme, identified [Wanders et al., 1990a] and purified [Van Veldhoven et al., 1992] from rat liver, has a molecular mass of 420 kDa and consists of identical subunits of 70 kDa. 3) a non-inducible trihydroxycoprostanoyl-CoA oxidase, only present in liver, oxidizes the CoA-esters of the bile acid intermediates, di- and trihydroxycoprostanic acid [Van Veldhoven et al., 1992]. In human liver peroxisomes only two acyl-CoA oxidases have been shown to be present [Vanhove et al., 1993b]: 1) a palmitoyl-CoA oxidase, which cross reacts with polyclonal antibodies to rat palmitoyl-CoA oxidase and showed similarity in molecular mass and subunit composition and oxidation of substrates with rat palmitoyl-CoA oxidase [Osumi et al., 1980] and 2) a branched-chain acyl-CoA oxidase, which did not cross react with polyclonal antibodies to rat palmitoyl-CoA oxidase and pristanoyl-CoA oxidase. The enzyme has been purified as a monomeric 70 kDa protein and oxidizes 2-methyl-branched chain acyl-CoAs as well as the bile acid intermediates, di- and trihydroxycoprostanic acid [Vanhove et al., 1993b].
Figure 11. Proposed structure of the peroxisomal fatty acid β-oxidation machinery. The figure is modified from Fig. 3 of Wanders et al. (1997). Abbreviations used: PM, peroxisomal membrane; THCA-CoA, trihydroxycholestanoyl-CoA; MFE1, multifunctional enzyme 1, also called pM0P1 (peroxisomal multifunctional β-oxidation protein 1) [Wanders et al., 1997] and bifunctional protein [Osumi et al., 1980]; MFE2, multifunctional enzyme 2, also known as pMOP2 (peroxisomal multifunctional β-oxidation protein 2) [Wanders et al., 1997], 17ß-hydroxysteroid dehydrogenase IV [Leenders et al., 1996], multifunctional protein 2 (MFP2) [Dienaidenoubhani et al., 1996], peroxisomal multifunctional enzyme II (per MFE-II) [Qin et al., 1997] and D-specific bifunctional protein [Jiang et al., 1997].

For a long time it was assumed that the second and third step of peroxisomal β-oxidation is catalyzed by one single inducible multifunctional protein [Osumi and Hashimoto, 1979b], displaying 2-enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase activity and additional by A3,A2,-enoyl-CoA isomerase activity required for unsaturated fatty acid β-oxidation [Palosaari et al., 1991]. This enzyme has been purified and characterized from rat liver [Osumi et al., 1985] and human liver [Reddy et al., 1987]. Since Cook and coworkers [Cook et al., 1992] described a hydratase 2 in rat liver having D-specific 3-hydroxyacyl-CoA dehydrogenase properties, it has been suggested that the
physiological role of this enzyme in mammals is to participate not in the metabolism of unsaturated fatty acids but in the metabolism of other derivatives of long-chain carboxylic acids, for instance, α-methyl branched chain acyl-CoA esters [Hiltunen et al., 1996].

Indeed, today it has become clear (fig 11) that two multifunctional enzymes (MFEs) exist in mammals, MFE1 and MFE2 which not only catalyze two reactions of opposite chiral specificity, but they also have different native molecular sizes and distinct amino acid sequences, indicating different phylogenetic origins. MFE1 is involved in the hydration of trans-enoic-CoAs to their L-hydroxy form, whereas MFE2 generates the D-hydroxy form [Qin et al., 1997]. Furthermore, different substrate specificities exist with MFE1 responsible for the formation of 3-ketoacyl-CoAs of long and very long-chain saturated and unsaturated fatty acids whereas MFE2 acts on pristanic acid and di- and trihydroxysterol esters [fig 11] [Dieuaideanoubhaniet al., 1996; Jiang et al., 1997]. The final step in peroxisomal β-oxidation is catalyzed by 3-ketoacyl-CoA thiolase, which is known to be synthesized as a precursor protein containing an amino-terminal pre-sequence (PTS 2). In rat, two genes (A, non-inducible and B, inducible) for peroxisomal 3-ketoacyl-CoA thiolase have been identified [Hitikata et al., 1987] with only slightly different amino acid sequences. Only the inducible 3-ketoacyl-CoA thiolase has been purified [Miyazawa et al., 1981] and consists of two identical 41 kDa subunits. In the human genome only one 3-ketoacyl-CoA thiolase gene exhibiting high structural similarity with the rat thiolase genes has been identified [Bout et al., 1991]. For a long time 3-ketoacyl-CoA thiolase was thought to be the only enzyme involved in the thiolytic cleavage of all 3-ketoacyl-CoAs formed in peroxisomes. Seedorf and coworkers [Seedorf et al., 1994] have characterized a peroxisomal sterol carrier protein x (SCPx) from rat liver, which consists of a N-terminal domain of 404 amino acids with intrinsic 3-ketoacyl-CoA thiolase activity and a 143-residue C-terminal domain with lipid-transferring activity. We have recently found that the 3-ketoacyl-CoA esters of pristanic acid and the hydroxycholestanoic acids are only handled by this 58 kDa SCPx [Wanders et al., 1997] (figure 11).

Unsaturated fatty acids can be β-oxidized in mitochondria and peroxisomes at rates dependent on the chain length of the fatty acids and the number and configuration of the double bonds. A characteristic of the auxiliary enzymes of β-oxidation is the occurrence of multiple isoforms not only in mitochondria and peroxisomes but also within the same organelle. This has been shown for 2,4-dienoyl-CoA reductase [Hakkola and Hiltunen, 1993], Δ3,Δ2-enoyl-CoA isomerase [Kilsen et al., 1992] and probably 2-enoyl-CoA hydratase 2 [Cook et al., 1992].

During β-oxidation of unsaturated fatty acids carrying a double bond extending from an even-numbered carbon atom, a 2,4-dienoyl-CoA is formed, which cannot be further degraded. First, 2,4-dienoyl-CoA is reduced to 3-trans-enoyl-CoA by a NADPH-dependent 2,4-dienoyl-CoA reductase [Dommes et al., 1981], thereafter it is isomerized by Δ3,Δ2-enoyl-CoA isomerase [Kärki et al., 1987] to form 2-trans-enoyl-CoA, which in turn can be oxidized normally in the β-oxidation cycle (figure 12).
Odd numbered double bonds can be removed by a NADPH dependent reduction once they are in the Δ5- position as a result of chain-shortening. This occurs in a four step reaction sequence resulting in a shift of the double bond from the Δ5- to the Δ2-position. The starting metabolite in this reaction sequence is 2-trans-5-cis-dienoyl-CoA, which is formed from 5-cis-enoyl-CoA by acyl-CoA dehydrogenase. 2-trans-5-cis-dienoyl-CoA can either complete the β-oxidation cycle to yield 3-cis-enoyl-CoA or can be converted to 3,5-cis-dienoyl-CoA by enoyl-CoA isomerase. Δ5,Δ2-dienoyl-CoA isomerase, [Luo et al., 1994] catalyzes the shift of both double bonds to produce 2-trans,4-trans-dienoyl-CoA which is reduced by 2,4-dienoyl-CoA reductase in a NADPH dependent manner to 3-trans-enoyl-CoA. Enoyl-CoA isomerase converts 3-trans-enoyl-CoA to 2-trans-enoyl-CoA (figure 12). These pathways are now known as the isomerase-dependent pathway, which only requires enoyl-CoA isomerase, and the reductase-dependent pathway which requires dienoyl-CoA isomerase, 2,4-dienoyl-CoA reductase and enoyl-CoA isomerase [Hiltunen et al., 1996]. Since dienoyl-CoA isomerase ensures the removal of 1,5-dienoyl-CoAs, it may be present in mitochondria and peroxisomes as a detoxification enzyme.

1.4.5 Differences between mitochondrial and peroxisomal β-oxidation

Despite the similarity in reaction mechanism, the mitochondrial and peroxisomal β-oxidation systems differ in several aspects. As has been discussed before the enzymes involved in mitochondrial and peroxisomal β-oxidation are different proteins encoded by different genes. The first difference is the first oxidation step. In mitochondrial β-oxidation this step is catalyzed by an acyl-CoA dehydrogenase which conserves the energy produced, by forming FADH2 which can flow into the respiratory chain leading to oxidative phosphorylation and high conserved energy as ATP. Furthermore high energy is conserved in the third step leading to NADH which again enters the respiratory chain. In contrast, in peroxisomal β-oxidation the first oxidation step involves an oxidase which releases its energy in the form of H2O2 which is lost as heat (possible role in thermogenesis). Some preservation takes place at the third oxidation step where energy is conserved in the form of NADH, which indirectly, via proposed shuttle mechanisms,
flows into the mitochondrial respiratory chain leading to formation of ATP [Baumgart et al., 1996a]. When comparing these two systems in light of energy conservation it is obvious that mitochondrial β-oxidation is energetically much more favourable than peroxisomal β-oxidation. Step one and three in mitochondrial β-oxidation results in two and three molecules of ATP, respectively.

The second difference is the kind of substrates that are oxidized in both organelles. Although there exists an overlap in substrate spectra, as discussed before, it has become clear that peroxisomes are only involved in very-long-chain fatty acid oxidation mainly because mitochondria lack the synthetase activity. Furthermore prostaglandins, 2-methyl branched chain fatty acids and the bile acid intermediates predominantly are oxidized in peroxisomes. It has been pointed out that CPT I probably plays a regulatory role in this, since this enzyme is hardly active towards these compounds. Conversely, short chain fatty acids are poorly or not at all oxidized by peroxisomes whereas mitochondria readily oxidize these compounds [Reddy and Mannaerts, 1994]. As mentioned by Reddy and Mannaerts, one has to keep in mind that the contribution of mitochondria and peroxisomes to substrate oxidation in the intact cell depends not only on the capability of each organelle to oxidize a particular substrate, but also on the substrate affinity, the specific β-oxidizing activity and the relative abundance of each organelle [Reddy and Mannaerts, 1994].

The third difference is that peroxisomal β-oxidation does not go to completion in contrast to mitochondrial β-oxidation. The substrates undergo a limited number of β-oxidation cycles [Lazarow, 1978] dependent on their chain length but it remains uncertain what the exact regulation of this process is. It has been observed that the acyl-CoA oxidase has little activity towards fatty acids with a chain length <C8. Furthermore, regulation can occur at the level of the acyl-CoA hydratase and carnitine octanoyl transferase due to competition for substrate. The chain-shortened fatty acids can either be transported to the mitochondria for further oxidation or can be esterified to glycerolipids in the ER.

The fourth difference exists in the fate of acetyl-CoA (in case of straight-chain fatty acid oxidation) and propionyl-CoA (in case of 2-methyl branched-chain fatty acid oxidation). Since peroxisomes do not have a Krebs cycle, the acetyl-CoA and propionyl-CoA cannot be oxidized within the peroxisomes but have to be transported to the mitochondria for further oxidation, or can be used for biosynthetic purposes. In chapter 5 we provide evidence at least in case of pristanic acid oxidation that the first peroxisomal β-oxidation product propionyl-CoA is transported as propionyl-carnitine to mitochondria for further oxidation to CO₂ in the Krebs cycle.

1.5 Concluding remarks and future prospects:
At the start of the investigations described in this Ph.D. thesis it was unclear what the role of peroxisomes in fatty acid oxidation was and how the endproducts of peroxisomal β-oxidation were shuttled to mitochondria for oxidation to CO₂ and H₂O. Indeed, according to some authors oxidation of very-long-chain fatty acids like C24:0 was mitochondrial whereas others suggested a primarily peroxisomal site of oxidation. In the former studies use was
made of inhibitors notably of the respiratory chain to discriminate between mitochondrial and peroxisomal β-oxidation.

As described in Chapter 2 and 3 such an approach is full of pitfalls and neglects the effects of these inhibitors on parameters such as cellular ATP levels and the cytosolic redox-state which affects peroxisomal β-oxidation. Our studies indicate that mitochondrial respiratory chain inhibitors cannot be used for this purpose. For this reason we searched for a selective inhibitor with the following characteristics:

1. Selective inhibition of a single enzyme or transporter.
2. No effect on cellular ATP levels and/or the redox-state.

2-[5-(4-chlorophenyl)pentyl]-oxirane-2-carboxylate (POCA), a selective inhibitor of CPT I, was chosen for this purpose. Titrations with POCA clearly established that oxidation of very-long-chain fatty acids like C26:0 is primarily if not exclusively initiated in peroxisomes. Using a different approach in which use was made of ethidium bromide treated cells devoid of oxidative phosphorylation, a similar conclusion was reached with octanoate and palmitate oxidation primarily in mitochondria and oxidation of C26:0 and pristanic acid predominantly in peroxisomes.

The results of Chapter 2-4 clearly show that EtBr-treated cells and especially POCA-treated cells can be used: 1. to dissect the contribution of peroxisomes and mitochondria to the β-oxidation of a certain fatty acid and 2. to follow the fate of the endproducts of peroxisomal β-oxidation. When these studies were being performed, it also became clear to us that mutant cells with selective defects in either mitochondrial or peroxisomal β-oxidation offer a unique opportunity to resolve the functional interaction between the mitochondrial and peroxisomal β-oxidation systems. This approach was used in Chapter 5 to study how the propionyl-CoA as released in the peroxisomal matrix after one cycle of β-oxidation, moves to the mitochondrion to be oxidized to CO₂ and H₂O, making use of fibroblasts from a patient with a genetic deficiency of the mitochondrial carnitine/acylcarnitine carrier. The results show that formation of CO₂ was completely blocked in these mutant cells showing that propionyl-CoA must enter the mitochondrion as propionylcarnitine via the carnitine/acylcarnitine carrier.

Subsequent studies (Chapter 6) provided conclusive evidence in favor of propionylcarnitine as obligatory intermediate. The results of Chapter 2-6 clearly show that fatty acids like pristanic acid and C26:0 undergo a number of cycles of β-oxidation in the peroxisome and that the endproducts such as acetyl-CoA and propionyl-CoA move to the mitochondria in the form of carnitine-esters. This is probably also true for medium-chain acyl-CoA esters both unbranched (coming from C26:0) and branched (coming from pristanic acid).

A number of questions remain:

1. What is the mechanism by which fatty acids like C26:0, pristanic acid, di- and trihydroxycholestanoic acid, dicarboxylic acids etc. enter the peroxisome?

Available evidence suggests that carnitine is not involved in the peroxisomal uptake of fatty acids. On the other hand it is clear now that the products of the gene defective in X-linked adrenoleukodystrophy, is somehow involved in the
uptake of very-long-chain fatty acids. The ALD-protein is a peroxisomal membrane protein belonging to the family of ABC-transporters. Our recent studies performed by Verleure et al. (1997) provide conclusive evidence that at least in the yeast S. cerevisiae transport of fatty acids across the peroxisomal membrane occurs in the form of CoA-esters as mediated by the yeast homologue of the ALD-gene.

Interestingly, recent studies have shown that peroxisomes contain at least four different ABC-transporters which may well play a role in the uptake of the various fatty acyl-CoAs into peroxisomes. In this respect it is important to mention that mice in which the gene coding for the third peroxisomal ABC-halftransporter (PMP70) is deficient, show profound dicarboxylic aciduria. On the other hand, peroxisomes were normal indicating that PMP70 is not involved in peroxisome biogenesis per se as originally concluded by Gärtner and Valle [1992]. We favor the hypothesis that PMP70 either as a homo- or heterodimer catalyzes the uptake of dicarboxyl-CoAesters into peroxisomes following their formation at the ER-membrane.

2. What is the mechanism by which carnitine-esters leave the peroxisome? Our findings in Chapter 5 and 6 that acetyl-CoA and propionyl-CoA enter the mitochondria as carnitine esters, suggest that acetyl-CoA and propionyl-CoA leave the peroxisome as carnitine-esters. This immediately explains now why peroxisomes contain carnitine acetyltransferase (CAT) and carnitine octanoyltransferase (COT) activity.

It is intriguing to know by which mechanism these carnitine-esters leave the peroxisome. Is there a peroxisomal carnitine/acylcarnitine carrier analogous to the mitochondrial carnitine/acylcarnitine carrier? Or is transport mediated by a completely different transporter? This is difficult to resolve in the experimental systems used in this thesis work. A good approach would be to use yeast as a model-system to resolve this question. This is now done by Carlo van Roermund who has isolated a large collection of yeast-mutants with no ability in peroxisome biogenesis but a selective defect in oleate β-oxidation which leads to an impaired ability to grow on oleate. Systematic analysis of all these mutants should lead to the identification of all steps involved in the oxidation of oleate to CO₂ and H₂O which must lead to the identification of the presumed carrier.