Review

Transport of fatty acids and metabolites across the peroxisomal membrane

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Abstract

The peroxisomal membrane forms a permeability barrier for a wide variety of metabolites required for and formed during fatty acid β-oxidation. To communicate with the cytoplasm and mitochondria, peroxisomes need dedicated proteins to transport such hydrophilic molecules across their membranes. Genetic and biochemical studies in the yeast *Saccharomyces cerevisiae* have identified enzymes for redox shuttles as well as the first peroxisomal membrane transporter. This peroxisomal ATP-binding cassette transporter (Pat) is highly homologous to the gene mutated in X-linked adrenoleukodystrophy (X-ALD). The yeast Pat is required for import of activated fatty acids into peroxisomes suggesting that this is the primary defect in X-ALD. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Fatty acids are important molecules for cellular life. They are efficient fuel molecules that produce a high yield of ATP upon degradation in the β-oxidation pathway. Esterified to glycerol or sterols, they constitute a depot of energy production in case of need. They are building blocks for lipids that constitute membranes bordering the various cellular compartments. They are structural elements of intracellular messengers such as diacylglycerol and phosphoinositides, and they are important effector molecules that regulate metabolism. Unsaturated fatty acids are precursors to eicosanoid hormones like leukotrienes, prostaglandins and thromboxanes. Finally, certain proteins are posttranslationally modified by covalent attachment of fatty acids like palmitate and myristate. The addition of such a hydrophobic group to a protein can influence its intracellular localisation and biological role.

The multiple use and fate of fatty acids in a cell make it difficult to study aspects of fatty acid biochemistry without interference from their other functions. For instance, in mammalian cells, degradation of fatty acids takes place in two different cellular compartments: peroxisomes and mitochondria. Very-long-chain fatty acids (VLCFAs) are first shortened in peroxisomes and the resulting long-chain

Abbreviations: LCFA, long-chain fatty acid (C16–C24); MCFA, medium-chain fatty acid (C8–C14); VLCFA, very long-chain fatty acid (C26); UFA, unsaturated fatty acid; VLACS, very-long-chain acyl-CoA synthetase; FATP, fatty acid transport protein; FAA, fatty acid activator; PAT, peroxisomal ATP-binding cassette transporter; X-ALD, X-linked adrenoleukodystrophy

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fatty acids (LCFAs) are then handed over to mitochondria for completion of oxidation. Basically, this requires a double set of \( \beta \)-oxidation enzymes (iso-enzymes) for each organelle with subtle differences in substrate specificity and co-factor requirements. Therefore, the interpretation of experiments in fatty acid biochemistry carried out in mammalian cells is often difficult.

For studies on peroxisomal fatty acid \( \beta \)-oxidation, *Saccharomyces cerevisiae* is a suitable model system. Certain aspects of mammalian fatty acid biochemistry are absent in yeast and fatty acid degradation is completely confined to peroxisomes; mitochondrial iso-enzymes are therefore absent. This facilitates setting up clean genetic and biochemical experiments.

Here we will review how fatty acids are taken up into yeast cells and prepared for import into peroxisomes from the cytoplasm prior to their oxidation. In addition, we will discuss supplementary processes required to keep peroxisomal \( \beta \)-oxidation going: export of the final product, acetyl-CoA, and the removal of reduction equivalents that accumulate during \( \beta \)-oxidation. An attempt will be made to relate results of fundamental studies in yeast to diseases in man.

2. Fatty acid metabolism in *S. cerevisiae*

In *S. cerevisiae*, the cellular fatty acid pool is fed by (1) long-chain fatty acyl-CoAs (palmitoyl-CoA and stearoyl-CoA) formed de novo by the concerted action of two enzyme systems, acetyl-CoA carboxylase (Acc1p) and fatty acid synthetase (FAS), (2) mobilisation of endogenously stored fatty acids from lipids (triacylglycerols, phospholipids, and sterol esters) and (3) uptake of exogenous fatty acids. The de novo synthesis of fatty acids yields acyl-CoA esters, the activated form of fatty acids that can be further metabolised. Free fatty acids, formed either by mobilisation from endogenous lipids or by uptake of exogenous fatty acids, need to be activated to acyl-CoA esters prior to further metabolism (Fig. 1). Acyl-CoA esters can be utilised for a variety of processes including fatty acid desaturation or elongation, lipid synthesis, protein acylation and energy production via peroxisomal \( \beta \)-oxidation [1]. *S. cerevisiae* can utilise exogenous LCFAs as sole carbon and energy source or when de novo fatty acid synthesis is blocked. This has allowed genetic approaches to identify factors that are required for uptake, activation and degradation of fatty acids. Below we will describe our current knowledge about the processes that facilitate utilisation of exogenous fatty acids for \( \beta \)-oxidation in *S. cerevisiae*.

3. Fatty acid transport across the plasma membrane

The question of whether fatty acids can move freely across a lipid bilayer or whether proteins are essential for their transport is one of long-standing interest, but is still controversial [2,3]. In vitro studies have shown that unionised fatty acids or derivatives thereof can cross an artificial phospholipid bilayer rapidly (\( t_{1/2} < 1 \) s) [4]. Various factors can influence the rate of passive fatty acid movement across a membrane, including: (1) a transmembrane pH gradient [5]; (2) the relative distribution of fatty acid binding sites on both sides of the membrane [3,6]; (3) modification of free fatty acids to membrane-impermeable derivatives (acyl-CoA esters) on the trans side of the membrane [7–9]; (4) utilisation of fatty acids...
acids for anabolic and catabolic processes, thereby creating a metabolic trap.

Although net fatty acid transport can occur by simple diffusion across the plasma membrane if combined with a mechanism which retains fatty acids or their derivatives in the cytosol, some investigators found saturable uptake of fatty acids which suggested to them a protein-mediated transport mechanism (reviewed in [2]).

Two classes of proteins have been identified that stimulate uptake of fatty acids. The first class includes proteins that bind fatty acids with high affinity and these proteins are thought to function as either extracellular or intracellular fatty acid receptors, depending on their subcellular localisation (reviewed in [2,6]). Both the relative distribution of fatty acid binding proteins over the plasma membrane and their affinities for fatty acids or fatty acid derivatives might drive net uptake. The second class includes acyl-CoA synthetases. Indeed, in *Escherichia coli*, mammalian cells and yeast, mutant cells lacking acyl-CoA synthetase activity are severely affected in fatty acid uptake [7,8,10,11]. Using an expression cloning strategy for proteins that increase uptake of a metabolisable fluorescent long-chain fatty acid analogue, Schaffer and Lodish [9] identified the long-chain fatty acyl-CoA synthetase (FACS) and a novel fatty acid transport protein (FATP) [9]. This integral plasma membrane protein belongs to a protein family which is conserved from mycobacterium to man [12] and which in mice consists of five members (FATP1–5). Overexpression of either murine FATP1, 2 or 5 or a *Caenorhabditis elegans* FATP homologue in COS cells was shown to increase uptake of the metabolisable fluorescent long-chain fatty acid analogue [12]. An *S. cerevisiae* FATP homologue, Fat1p, was initially reported to contribute to oleate uptake at low oleate concentrations [13]. However, several observations suggest that fat1Δ cells are disturbed in a fatty acid-metabolising process which secondarily results in a decreased fatty acid uptake. For instance, in cells that are not able to synthesise fatty acids de novo, Fat1p is required for oleate utilisation. Under these experimental conditions, LCFA uptake was not disturbed [11]. Moreover, it is hard to envisage a direct role in fatty acid uptake for a protein that has been found associated with peroxisomes and the endoplasmic reticulum [11,14]. Furthermore, very-long-chain fatty acyl-CoA synthetase (VLACS) activity is decreased in fat1Δ cells and they accumulate VLCFAs intracellularly. These observations suggest that Fat1p is required for activation of endogenous VLCFAs prior to their breakdown via fatty acid β-oxidation [11,14]. Its partial association with peroxisomes is in agreement with such a function. Interestingly, studies in mammals revealed a similar connection between the FATP protein family and acyl-CoA synthetases. Hashimoto and co-workers previously purified the rat homologue of FATP2 as an enzyme with VLACS activity from liver peroxisomes [15,16]. Overexpression of rat FATP2 in COS cells increased VLACS activity [16]. The role of acyl-CoA synthetases in fatty acid uptake might be by converting membrane-permeable fatty acids to membrane-impermeable acyl-CoA esters, a process named vectorial acylation [7]. Alternatively, some FATP family members might have a dual function whereby they couple fatty acid transport and fatty acid activation in analogy to the glucose transporter and hexokinase.

### 4. Activation and intracellular distribution of fatty acids

As mentioned above, upon uptake, fatty acids are activated prior to further metabolism. The *S. cerevisiae* genome contains six open reading frames (ORFs) that show amino acid sequence homology to acyl-CoA synthetases. Three of these fatty acid activators (Faa1p, Faa2p and Faa3p) have been shown to exhibit acyl-CoA synthetase activity with a partial overlapping substrate specificity in vitro [17]. However, these various acyl-CoA synthetases are not functionally interchangeable in vivo. For instance, Faa1p and Faa4p are required for uptake and further metabolism of LCFA [18] and Faa2p, a peroxisomal enzyme, is required for β-oxidation of medium-chain fatty acids (MCFAs) [19]. Fat1p is a VLACS that is required for proper maintenance of VLCFA levels. Fat1p probably activates VLCFAs, released from structural lipids, for degradation via the β-oxidation pathway [11,14]. The in vivo role of Faa3p and Fat2p is unclear [20,21]. The subcellular localisation and the substrate specificity of the acyl-CoA synthetases determine where a particular
fatty acid is activated and what its metabolic fate will be. For instance, MCFAs are activated mainly by the peroxisomal enzyme Faa2p, indicating that MCFAs migrate through the cytoplasm either as free fatty acids or bound to protein. Furthermore, peroxisomal activation of MCFAs destines them for degradation by \( \beta \)-oxidation. Since Faa2p has been shown to activate LCFAs in vitro it could in principle direct LCFAs for peroxisomal degradation. However, in vivo, LCFAs are thought to be activated upon cell entry and can then be subdivided over various LCFA-consuming processes (Fig. 1). Peroxisomes rely on a specialised uptake system to tap the cellular acyl-CoA pool (see below).

High concentrations of free fatty acids and acyl-CoA esters are detrimental to membranes. However, in the cytoplasm acyl-CoA binding proteins and fatty acid binding proteins (ACBPs and FABPs) keep the actual free acyl-CoA and fatty acid concentration very low. Besides protecting the cellular membranes from fatty acids or their derivatives, FABPs and ACBPs have been postulated to play a role in the delivery of fatty acids and acyl-CoA esters to various compartments and fatty acid-consuming systems [22–24].

5. Protein-mediated transport of long-chain fatty acids across the peroxisomal membrane

Continuous \( \beta \)-oxidation depends on the availability of acyl-CoA, NAD\(^+\), NADPH, free coenzyme A and on export of acetyl groups (Fig. 1). The peroxisomal membrane forms a selective permeability barrier in vivo (reviewed by [25]), implying the existence of specialised metabolite transport systems.

We reasoned that defects in the systems that circumvent the putative peroxisomal membrane barrier would result in the following characteristics in \( S.\ cerevisiae \): (1) a defect in the ability to use fatty acids for growth; (2) a loss of the ability to degrade radiolabelled fatty acids in intact cells; (3) a retention of the ability to degrade radiolabelled fatty acids after cell lysis provided that the assay mixture is supplemented with cofactors. This principle of latency has helped us to identify various factors required for \( \beta \)-oxidation, substrate import, product export and cofactor regeneration [19,26,27].

LCFAs are activated outside peroxisomes, implying that it is not free LCFAs but their activated derivatives that are transported across the peroxisomal membrane [19,28,29]. In vitro studies have shown that in contrast to olate, oleoyl-CoA does not spontaneously flip from one leaflet to the other leaflet of lipid vesicles [30]. This implies that a specialised import system is required for import of activated fatty acids across the peroxisomal membrane.

X-Linked adrenoleukodystrophy (X-ALD) patients accumulate VLCFAs due to a defect in a peroxisomal ABC transporter [31,32]. Therefore, by inference, ALDp could be involved in transport of VLCFAs across the peroxisomal membrane. The yeast genome encodes two peroxisomal ABC half-transporters (Pat1p and Pat2p also known as Pxa2p and Pxa1p) which show extensive amino acid sequence similarity to ALDp [33–35]. Pat1p and Pat2p are induced upon growth on oleate and are necessary for normal growth on LCFAs as sole carbon source. Genetic experiments suggested that Pat1p and Pat2p function together as one transporter [19,36]. Indeed, co-immunoprecipitation experiments have shown that Pat1p and Pat2p form a heteromeric complex [36]. LCFA \( \beta \)-oxidation activity in intact cells of \( PAT1 \) and \( PAT2 \) deletion mutants was reduced to approx. 20% of the wild type level. However, \( \beta \)-oxidation in detergent lysates was unaffected. This indicates a role for Pat1p/Pat2p in a transport step of LCFA \( \beta \)-oxidation. Since the Pat1p/Pat2p complex is only required for \( \beta \)-oxidation of LCFAs activated outside peroxisomes whereas fatty acids that are activated inside peroxisomes are \( \beta \)-oxidised independently from Pat1p/Pat2p (see Fig. 2 and below), the peroxisomal ABC transporter is probably transporting the activated fatty acids [19,37]. \( S.\ cerevisiae \) seems to lack carnitine palmitoyltransferase (CPT) activity [38]. Furthermore, recent identification of the rat mitochondrial acylcarnitine/carnitine translocator shows no sequence homology with Pat1p/Pat2p [39]. These results imply that transport of LCFAs across the peroxisomal membrane differs from that across the mitochondrial membranes in mammals, where activated fatty acids are translocated as carnitine esters via the carnitine/acylcarnitine translocator.

Translocation of phospholipids across the plasma membrane of mammalian cells has been shown to be
dependent on the action of the ABC transporters Mdr1p and Mdr3p [40,41]. These proteins have been postulated to function as flippases that bind an amphipathic lipid in the cytoplasmic leaflet of the plasma membrane and flip its polar group across the membrane to deliver the molecule to the extracellular leaflet of the plasma membrane. Subsequent β-oxidation of the acyl-CoA esters inside peroxisomes will allow a net flux of long-chain acyl-CoA esters into peroxisomes. In this way, peroxisomes can tap the cytoplasmic long-chain fatty acyl-CoA pool for β-oxidation and compete with other long-chain fatty acyl-CoA-consuming processes.

6. Transport of medium-chain fatty acids across the peroxisomal membrane

MCFAs might traverse the plasma membrane by simple diffusion. Crucial to the understanding of MCFA transport through the cell was the observation that almost all MCFA-CoA synthetase activity is present inside peroxisomes [19]. Thus, after entering the cytoplasm, MCFAs might diffuse through the cytoplasm either as free fatty acids or bound to fatty acid-binding proteins. MCFAs might then traverse the peroxisomal membrane either by diffusion and spontaneous flipping to the inner leaflet of the peroxisomal membrane or by protein-mediated transport. Upon entry into peroxisomes, MCFAs become activated by Faa2p and are subsequently β-oxidised [19]. The intraperoxisomal activation could set up a gradient to allow passive diffusion of MCFAs across the peroxisomal membrane. MCFA β-oxidation occurs independently of Pat1p/Pat2p. Therefore, both the chain length and the subcellular site of activation determine whether a fatty acid depends on Pat1p/Pat2p for its β-oxidation. As mentioned above, mis-targeting of Faa2p to the cytoplasm by deletion of its peroxisomal targeting signal results in a Pat dependence for C12:0 β-oxidation.

7. Additional transport events across the peroxisomal membrane

As mentioned before, mammalian peroxisomes do
not β-oxidise fatty acids to completion. MCFA-CoA esters have been shown to be converted to carnitine esters that leave peroxisomes and enter mitochondria. Reconversion to MCFA-CoA on the luminal side of the mitochondrial inner membrane allows further oxidation to acetyl-CoA. On the other hand, β-oxidation of fatty acids in yeast is restricted to peroxisomes and can occur to completion. However, there are some indications that fatty acids are chain-shortened to myristate (C14:O) inside peroxisomes and can subsequently leave peroxisomes to be used in the cytoplasm for protein N-myristoylation in both yeast and mammals [18,44]. How this transport is mediated is unclear but the presence of an abundant peroxisomal thioesterase in S. cerevisiae with a preference for C14-CoA (Kal and Tabak, unpublished observations) suggests that the membrane-impermeable C14-CoA is hydrolysed to the membrane-permeable C14:0. Free C14:0 might simply diffuse out of peroxisomes and can then be used in the cytoplasm for myristoylation.

The end product of fatty acid β-oxidation, acetyl-CoA, is not able to diffuse out of peroxisomes (Fig. 3); the C2 unit is either exported via a carnitine-dependent pathway to mitochondria or as glyoxylate cycle intermediates [26]. A block in either of these pathways alone does not inhibit β-oxidation; only a block in both pathways inhibits fatty acid β-oxidation. The glyoxylate cycle allows the formation of C4 carbon skeletons from C2 units.
During β-oxidation of fatty acids, NAD$^+$ is reduced to NADH. For continuation of β-oxidation, NADH must be re-oxidised to NAD$^+$. Enzymes of NAD$^+$-regenerating systems have been found in peroxisomes of mammals, plants and fungi. Evidence for a crucial role of intraperoxisomal NAD$^+$ regeneration came from van Roermund et al. [26]. Deletion of the MDH3 gene, encoding peroxisomal malate dehydrogenase, blocks fatty acid β-oxidation in intact cells and causes 3-hydroxyacyl-CoA accumulation. The Mdh3p-dependent regeneration of NAD$^+$ depends on the presence of oxaloacetate inside peroxisomes. Since continuous β-oxidation would use up oxaloacetate and form large quantities of malate, β-oxidation would stall. The most simple model for continuation of β-oxidation in vivo is that for each oxaloacetate that enters peroxisomes a malate is exported [25,26]. This putative malate-oxaloacetate shuttle system or a more complicated variant of it transfers electrons from peroxisomal NADH to the cytosol. It has been hypothesised that the reduction equivalents are shuttled via the aspartate-malate shuttle system or a more complicated variant of it that transfers electrons from peroxisomal NADPH to peroxisomal NADH‡.

8. Peroxisomal β-oxidation and disease

The importance of peroxisomes in human metabolism is exemplified by the occurrence of severe genetic disorders caused by an impairment of one or more peroxisomal functions. The most severe disorders, including Zellweger syndrome and neonatal adrenoleukodystrophy, are characterised by a general loss of peroxisome functions caused by a defect in the assembly of the organelle. In contrast to the extensively studied human mitochondrial β-oxidation system, where the genetic basis for many different single enzyme deficiencies has been resolved, research into the peroxisomal β-oxidation disorders is still in its infancy. This is mainly caused by a serious gap in the knowledge of the enzymes that constitute the peroxisomal β-oxidation system. To bridge this gap, a better description of the enzymes that mediate the various biochemical reactions and proteins that mediate transport of metabolites across the peroxisomal membrane is urgently needed. S. cerevisiae, as model system to study peroxisome biogenesis, has been very important in the identification of the genetic defects in the peroxisome biogenesis disorders [50]. By analogy, careful genetic dissection of the S. cerevisiae peroxisomal β-oxidation system will undoubtedly help to identify the enzymes and factors required for metabolite transport and cofactor regeneration in man. Furthermore, as discussed above, a detailed
analysis of the \(S.\) \textit{cerevisiae} peroxisomal fatty acid transport system has provided insight into the function of the protein affected in the most common peroxisomal disorder, X-ALD.

Mammalian peroxisomes \(\beta\)-oxidise a wide range of different substrates including VLCFAs and LCFAs, long-chain dicarboxylic acids, certain prostaglandins and leukotrienes, di- and trihydroxycholestanolic acid, pristanic acid and certain mono- and polyunsaturated fatty acids. Many of these substrates have been shown to be activated outside peroxisomes [51]. These activated substrates are most likely transported by either homo- or heterodimers of the four different peroxisomal ABC half-transporters that have been identified thus far. Three of these peroxisomal ABC half-transporters are partially redundant [52], suggesting that they transport a partially overlapping set of \(\beta\)-oxidation substrates. This knowledge may be beneficial in the design of a novel therapy for X-ALD. This therapy is based on the pharmacologically induced upregulation of the partially redundant peroxisomal ABC half-transporters in patients with defects in the X-ALD transporter [53].

9. Concluding remarks and remaining questions

Recent studies have established that the peroxisomal membrane forms a permeability barrier to hydrophilic molecules in vivo. These findings raised the question as to how substrates and products are exchanged between cytoplasm and peroxisomal lumen and how the availability of cofactors is regulated. Although some of the enzymatic components of redox shuttles and export shuttles have been identified, the actual transporters have remained elusive. An exception is the peroxisomal ABC transporter Pat1p/Pat2p. This transporter is required for peroxisomal uptake of activated fatty acids from the cytoplasm. Whether free fatty acids enter peroxisomes via simple diffusion or whether proteins are involved to stimulate peroxisomal entry or exit is unclear. The peripheral association of Faa2p with the inner leaflet of the peroxisomal membrane is intriguing and might reflect an interaction with such a putative fatty acid transport protein. Further insight into the transport mechanisms across the peroxisomal membrane has to come first from identification of the transporters and second from functional analysis of these proteins after in vitro reconstitution in defined membranes.

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