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Biochimica et Biophysica Acta 1763 (2006) 1527–1540

BIOCHIMICA ET BIOPHYSICA ACTA

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Review

The ins and outs of peroxisomes: Co-ordination of membrane transport and peroxisomal metabolism

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Received 23 June 2006; received in revised form 15 August 2006; accepted 18 August 2006
Available online 23 August 2006

Abstract

Peroxisomes perform a range of metabolic functions which require the movement of substrates, co-substrates, cofactors and metabolites across the peroxisomal membrane. In this review, we discuss the evidence for and against specific transport systems involved in peroxisomal metabolism and how these operate to co-ordinate biochemical reactions within the peroxisome with those in other compartments of the cell.

Keywords: ABC transporter; β-oxidation; Adenine nucleotide translocator; Redox shuttle; Peroxisomal pH; Porin

1. Introduction

Peroxisomes are near-ubiquitous organelles of eukaryotic cells which perform a range of functions, many of which are organism- or tissue-specific [1,2]. The common metabolic activities of peroxisomes are the oxidation of fatty acids and generation and removal of hydrogen peroxide, but peroxisomes have also been implicated in β-oxidation of aromatic and cyclic compounds, synthesis of plasmalogens, isoprenoids, penicillin, lysine and glycine betaine, metabolism of purines and pyrimidines, and catabolism of polyamines, D-amino acids and methanol. Dependent on the organism, these organelles also participate in the glyoxylate cycle, pentose phosphate pathway and photorespiration and key roles for peroxisomes in signalling and development have emerged recently ([3,4], and references therein). This diversity of function is often reflected in peroxisomal specialisation, especially in plants [5] and also in trypanosomes [6,7].

The multiple roles of peroxisomes imply not only that a relatively large number of chemically diverse solutes must enter the peroxisome, but also that the products of peroxisomal metabolism must be exported from the organelle. Moreover, since a number of pathways are shared between the peroxisome and other cellular compartments, the metabolic steps that occur within the peroxisomal matrix or on the inner face of the peroxisomal membrane must be integrated with those in other cellular compartments to permit efficient operation of biochemical pathways.

The transport properties of the peroxisomal membrane have been a controversial issue for several decades. Early studies with isolated organelles revealed a lack of structure-linked latency for certain peroxisomal enzymes, suggesting that these organelles are freely-permeable to substrates (for more details see Antonenkov and Hiltunen, this issue). Although critics of this hypothesis have argued that the *in vitro* permeability of the membrane reflects damage and disruption during isolation (e.g. [1]), there have been a number of reports of relatively non-specific porin-like proteins in the peroxisomes of mammals, plants and yeast (reviewed in [8]). Recent, rigorous studies with isolated peroxisomes indicate that these organelles are permeable to low molecular weight, hydrophilic solutes such as glycolate and urate, but not to more bulky cofactors [9]. The latter conclusion is strongly supported by genetic studies which demonstrate that the peroxisomal membrane of *Saccharomyces cerevisiae* is impermeable to NAD(H) and acetyl-CoA *in vivo* [10]. Although the functions of many peroxisomal membrane proteins remain unknown, a growing number of peroxisomal

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doi:10.1016/j.bbamcr.2006.08.012

transport proteins have been characterised at the molecular level and these form the subject of this review. Of these, the best characterised are the adenine nucleotide translocator and ATP-binding cassette (ABC) transporters which have been implicated in transport of substrates for β-oxidation.

2. Import of substrates, co-substrates, and cofactors for β-oxidation

2.1. Import of substrates for β-oxidation: the role of ABC transporters

β-Oxidation not only provides a major pathway for the degradation of fatty acids but also serves a synthetic role in the production of a range of bioactive, lipid-derived molecules such as jasmonates, plasmalogens and bile acids [1,11]. In yeast and plants, the peroxisome is the sole site of fatty acid β-oxidation, whereas in mammals, very long chain fatty acids (VLCFA) are first chain-shortened in the peroxisome prior to complete oxidation in mitochondria. Oxidation of long chain fatty acids (LCFA) is predominantly mitochondrial and short- and medium chain fatty acids (S- and MCFA) are oxidised exclusively in mitochondria. In all cases, formation of fatty acyl-CoA esters is a prerequisite for entry into the β-oxidation spiral and is catalysed by members of a family of acyl-CoA synthetase enzymes, also known as activases, which differ in their substrate specificity. The intracellular location of fatty acid activation differs according to chain length and organism and thus determines the identity of the molecular species which must traverse the peroxisomal membrane. SCFA and MCFA are considered to be readily membrane-permeable and are able to enter mitochondria and peroxisomes in their protonated form. Esterification of free fatty acid to CoA, however, generates an amphipathic molecule, which is unable to cross membranes in the absence of a transport protein. Genetic studies have provided strong evidence for the involvement of members of the ABC protein superfamily in the peroxisomal import of substrates for β-oxidation [3].

A functional ABC protein comprises two copies each of two units: the membrane spanning domain (MSD), also known as the transmembrane domain (TMD) and the soluble nucleotide binding fold (NBF) [12]. In eukaryotes, these units are frequently contained within a single polypeptide with the topology TMD-NBF-TMD-NBF, an arrangement commonly referred to as a "full size" transporter. Alternatively, two "half size" transporters (TMD-NBF) may dimerise to form a functional ABC protein. Peroxisomal ABC transporters belong to subclass D, almost all members of which are half size transporters. The ABCD subfamily contains between one and five members and is represented in all eukaryotic taxa studied to date [3,13–22], but has been most extensively studied in mammals, baker's yeast and the model plant, *Arabidopsis thaliana*.

2.1.1. Yeast

Saccharomyces cerevisiae contains two peroxisomal ABC transporters, Pxa1p and Pxa2p (also known as Pat2p/Pal1p and Pat1p), which have been shown by genetic and co-immunoprecipitation studies to form a functional heterodimer in the peroxisomal membrane [23–26]. *pxa1*Δ and *pxa2*Δ deletion mutants are unable to grow on the LCFA oleate as the sole carbon source, but growth on MCFA such as laureate was unaffected, suggesting that these transporters play a specific role in β-oxidation of LCFA. This agrees with the finding that MCFA are imported into the peroxisome as free fatty acids where they are activated by the acyl-CoA synthetase Faa2p [23]. In contrast, LCFA are activated in the cytosol and LCFA-CoA esters must be transported across the peroxisomal membrane (Fig. 1). Biochemical studies with permeabilised spheroplasts provided evidence that Pxa2p is required for import of LCFA-CoA but not of SCFA-CoA [27] and mistargeting of Faa2p to the cytosol was shown to make β-oxidation of MCFA dependent on Pxa1p/Pxa2p [23], providing further support that this is an acyl-CoA transporter [28].

2.1.2. Plants

The genome of *Arabidopsis thaliana* contains a single ABCD gene, known as COMATOSE (CTS) and also as PXA1, PED3 and *At*ABCD1 [15,29,30]. Interestingly, unlike non-plant ABCD proteins, CTS is a full-length ABC transporter, in which two dissimilar half-size units are contained in a single polypeptide. All plant ABCD proteins identified to date have this modular architecture, suggesting that the fusion of two halves was a single, ancient event in evolutionary history. CTS is expressed ubiquitously in *Arabidopsis* and, unsurprisingly, null alleles have a pleiotropic phenotype: *cts* mutants fail to germinate in the absence of classical dormancy-breaking treatments and seedlings require sucrose for establishment [15,31]. Oil bodies and fatty acyl-CoAs, particularly VLCFA-CoAs, are retained in mutant seedlings, indicating an inability to break down storage lipid [15]. It has been suggested therefore, that CTS either transports or regulates the import of fatty acids or fatty acyl-CoAs into the peroxisome [3,11,15,29,30]. The transported species is still a matter for debate: whilst the retention of VLCFA-CoA is suggestive that CTS transports acyl-CoA, it is possible that the CoA pool represents a sink for free fatty acids and thus that CoA retention does not reflect cytosolic accumulation of CTS substrates. Moreover, the characterisation of a double peroxisomal acyl-CoA synthetase mutant provides evidence that free fatty acids could be transported into plant peroxisomes. *Arabidopsis* has two peroxisomal acyl-CoA synthetases (LACS 6 and 7) which have a substrate preference for (V)LFCA [32]. The active sites of LACS 6 and 7 are within the peroxisome and the *lacs6*, *lacs7* double mutant is unable to degrade storage lipid and cannot establish in the absence of sucrose [32,33]. Furthermore, peroxisome morphology of β-oxidation mutants such as *kat2* (thiolase) and *mfp2* (multifunctional protein) is distorted due to intraperoxisomal accumulation of acyl-CoAs [34,35], whereas it is normal in *cts* and *lacs6*, *lacs7*, suggesting that knocking out either the CTS transporter or the peroxisomal acyl-CoA synthetases prevents accumulation of acyl-CoAs in peroxisomes [36].

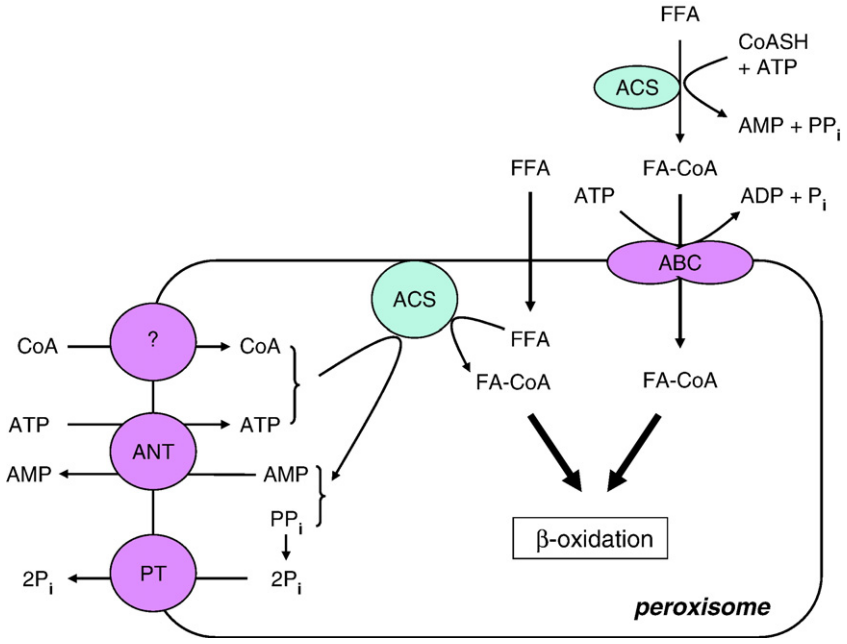

Fig. 1. Transport processes associated with β-oxidation. Dependent on chain length and organism, fatty acids are imported into peroxisomes as free acids (FFA) or CoA esters (FA-CoA). CoA esters formed in the cytosol or on the endomembrane system by the action of acyl-CoA synthetases (ACS) are transported across the peroxisomal membrane by one or more ABC proteins, at the expense of ATP hydrolysis. Alternatively, short- and medium chain free acids may enter the peroxisome by passive transport and are activated by peroxisomal ACS. This reaction requires intraperoxisomal pools of ATP and CoA which are supplied by the peroxisomal adenine nucleotide translocator (ANT) and by an unknown mechanism (?) respectively. AMP and pyrophosphate (PPi) are produced as by-products of fatty acid activation; AMP is exchanged for ATP by ANT and pyrophosphate is thought to be converted to inorganic phosphate which is exported by an NEM-insensitive phosphate transport protein (PT).

Other aspects of the *cts* mutant phenotype suggest that CTS could be a transporter of broad substrate specificity. Roots of *cts* mutants are resistant to indole butyric acid (IBA) and 2,4 dichlorophenoxybutyric acid (2,4-DB), indicating that they are unable to metabolise these compounds by β-oxidation to the corresponding active auxins [15,29,30]. The simplest explanation for these data is that CTS transports IBA and 2,4-DB or their CoA esters. Both basal and wound-induced jasmonic acid (JA) levels are reduced in *cts* mutants, suggesting that CTS is also involved in peroxisomal import of jasmonate precursors which yield JA following reduction by the peroxisomal enzyme OPR3 and three rounds of β-oxidation [37]. Many JA biosynthetic and signalling mutants are male sterile but *cts* mutants apparently have sufficient JA to retain fertility, suggesting an additional, probably passive, route for import of JA precursors. Nevertheless, fertilisation is reduced in *cts* mutants, which exhibit defects in pollen tube growth. This phenotype is not rescued by application of JA, suggesting an additional role for CTS in optimal fertility in *Arabidopsis* (S. Footitt, D. Dietrich, A. Baker, M. Holdsworth and F. Theodoulou, unpublished data). Taken together, comparisons of the phenotypes of *cts* mutants with those defective in other steps of β-oxidation is pointing to a role of β-oxidation not only in energy metabolism but also in the generation or processing of developmental signals and new roles for peroxisomal metabolism may await discovery in plants. An exciting challenge for the future will be the determination of the precise biochemical role of CTS, which awaits transport studies.

2.1.3. Mammals

The ABCD subfamily of humans and mice has four members: Adrenoleukodystrophy protein (ALDP; ABCD1), Adrenoleukodystrophy related protein (ALDR; ABCD2), PMP70 (ABCD3) and PMP69 (P70R; ABCD4), which exhibit overlapping but distinct tissue expression patterns [38–43]. These proteins have been shown to be integral peroxisomal membrane proteins [40,41,43–46] and a search for interacting partners revealed that ALDP, ALDR and PMP70 interact with PEX19, a cytosolic acceptor for peroxisomal membrane proteins which is involved in their intracellular sorting and trafficking to the peroxisomal membrane [47–50].

PMP70 was the first peroxisomal ABC transporter to be identified and characterised functionally and is expressed predominantly in liver [40], but transcripts have been detected in all tissues investigated [41]. Overexpression of rat PMP70 in Chinese hamster ovary cells led to an increase in LCFA but not VLCFA β-oxidation by peroxisomes and site-directed mutagenesis demonstrated a requirement for conserved motifs in ATP binding and hydrolysis [51]. Unlike many genes encoding peroxisomal proteins, mutations in *ABCD3* are not associated with disease in humans and the physiological significance of PMP70 awaits investigation [52,53]. Preliminary reports from *abcd3*−/− knockout mice suggest an additional role in transport of 2-methylacyl-CoA esters [54]. On the basis of sequence homology, PMP69 has been suggested to play a similar role to PMP70, though this transporter has not yet been characterised functionally [38].

ALDP has received much attention due to its role in the X-linked neurodegenerative disease, Adrenoleukodystrophy (X-ALD), which is characterised biochemically by the accumulation of VLCFA in plasma and tissue. Although X-ALD was originally associated with reduced activity of VLC acyl-CoA synthetase [55–57], positional cloning identified the *ALD* gene as an ABC transporter [42] and transfection with the wild type *ABCD1* cDNA was shown to correct the VLCFA β-oxidation defect of X-ALD fibroblasts [58–60]. Currently, over 650 different mutations in the *ABCD1* gene have been reported [52; www.X-ALD.nl]; in the majority of cases investigated, immunoreactive ALD protein is absent, suggesting the existence of an efficient retrieval mechanism for mutant forms of ALDP or that non-functional ALDP is unstable [46,61,62]. The precise relationship between the clinical symptoms of X-ALD, the biochemical phenotype and mutated ABCD1 is not currently clear [63,64]; for example, mouse models have been developed for X-ALD and although *abcd1−/−* mice replicate the biochemical defects of patients, they do not exhibit the clinical and pathological symptoms until very late in life [65–67]. It has been proposed that elevated VLCFA levels in X-ALD are related to mitochondrial dysfunction [68], although this has been contested by a detailed ultrastuctural study of mitochondria [69]. Enhanced microsomal fatty acid chain elongation has also been suggested as an additional mechanism underpinning VLCFA accumulation in X-ALD [70]. Despite the uncertainty regarding the pathology of X-ALD, it remains plausible that ALDP is a VLCFA or VLCFA-CoA transporter [52], and support for this hypothesis has been obtained by substrate-induced conformational changes [71], although unequivocal proof awaits direct demonstration of transport activity for purified ALDP.

Complementation studies have shown that expression of ALDRP and, surprisingly, PMP70 restores VLCFA β-oxidation in X-ALD fibroblasts, preventing VLCFA accumulation [58,63,72–74]. Pharmacological induction of endogenous peroxisomal transporters also resulted in restoration of VLCFA β-oxidation, providing promising leads for therapy [73,74]. These data suggest that mammalian peroxisomal ABC transporters are at least partially redundant and functional overlap between ALDP and ALDR has been confirmed by studies with knockout mice [75].

It has been suggested by several authors that the four half size transporters of the mammalian ABCD subfamily might heterodimerise to yield variants with modified transport properties [39,43,76], by analogy to human transporters associated with antigen processing (TAP) belonging to ABC subfamily B [77]. A number of studies have provided evidence for both homo- and heterodimerisation of ALDP, ALDR and PMP70 *in vitro* [63,78,79]. However, the expression patterns imply that, in several cell types, heterodimerisation is not physically possible *in vivo* [41,80,81] and this view is supported by the differential induction by pharmacological agents [82]. Moreover, purification of native ALDP and PMP70 strongly suggest that homomeric interactions predominate *in vivo* [83]. Nevertheless, heterodimerisation cannot be ruled out under specific conditions and is an important consideration for X-ALD gene therapy.

2.2. Transport of adenine nucleotides

The peroxisomal transporter best characterised is the adenine nucleotide transporter of *S. cerevisiae*, which is required for the activation of MCFA, the only established ATP-consuming reaction inside peroxisomes (Fig. 1). As discussed above, while the cytosolic fatty acid activation enzymes preferentially use LCFA as substrates, peroxisomes are the predominant site for MCFA activation [23]. The latter reaction is dependent on the supply of intraperoxisomal ATP and is catalysed by peroxisomal acyl-CoA synthetase Faa2p. Upon formation of the fatty acyl-CoA product, AMP is generated, which has to be exported back to the cytosol due to the lack of an adenylate kinase or an ATP regeneration system within peroxisomes. This cross-membrane traffic of adenine nucleotides (ATP in, AMP out) is catalysed by Ant1p [84,85], a member of the mitochondrial carrier family (MCF) generally involved in solute transport [86]. Both genetic and biochemical evidence supports this view: cells mutated in either *FAA2* or *ANT1* grow normally on LCFA but fail to do so on MCFA. In combination with a disabled Pxa1p–Pxa2p transporter, the *faa2*Δ and the *ant1*Δ mutants are unable to metabolise any fatty acid [23,84,85]. The β-oxidation rates of MCFA of an *ant1*Δ strain are also reduced when measured with intact cells. However, the respective cell lysates show a normal level of β-oxidation, which is consistent with a blockage in the ATP transport across the peroxisomal membrane in intact *ant1*Δ cells [85]. Likewise, heterologous expression of a peroxisomally targeted luciferase, which emits light in an ATP-dependent manner, required Ant1p for activity, but not when assayed in cell lysates [85].

Functional reconstitution of purified Ant1p into liposomes revealed the ability of Ant1p to transport adenine nucleotides specifically. ATP uptake was measured with proteoliposomes loaded with ATP, ADP, AMP, or the respective desoxynucleotides, whereas uptake was very low with other nucleotides or a number of other substrates for various known transporters [84]. The established inhibitors of the mitochondrial ADP/ATP carrier, carboxy atractyloside and bongkrekic acid [87] did not reduce the activity of Ant1p, indicating that Ant1p represents a novel type of MCF transporter. Interestingly, Ant1p exhibits two transport modes, not only the exchange described above, but also the uniport of adenine nucleotides [88]. *In vivo*, the former mode serves to exchange ATP for AMP under ongoing β-oxidation, whereas the latter is probably most important for loading peroxisomes with ATP early in their genesis (Fig. 1). Since both modes are proton-compensated and electroneutral [88], import of negatively charged ATP should contribute to the formation of a proton gradient across the peroxisomal membrane. Consistent with this idea, the ΔpH across the peroxisomal membrane is significantly diminished in an *ant1*Δ knock-out strain as measured by pH-sensitive variants of fluorescent proteins [88,89]. The significance of such measurements will be discussed below.

Pmp47p from *Candida boidinii* represents a functional homolog of Ant1p. Sakai and colleagues showed that a *pmp47*Δ mutant also exhibits a specific growth defect on MCFA, which can be rescued by introducing *S. cerevisiae* Faa2p

devoid of its peroxisomal targeting signal [90]. Growth on MCFA was also restored upon expression of cytosolic Faa2p in *S. cerevisiae ant1*Δ cells [85] providing evidence that Pmp47p and Ant1p function as peroxisomal ATP carriers. Given that MCFA are now activated by truncated Faa2p in the cytosol and transported into peroxisomes through Pxa1p–Pxa2p, intraperoxisomal ATP becomes dispensable. That heterologous expression of PMP47 is able to restore growth of the *S. cerevisiae ant1*Δ mutant on MCFA [84] lends further credence to the conception that Pmp47p represents the *C. boidinii* ortholog of Ant1p.

The human MCF member PMP34 localises to peroxisomes and its sequence is closely related to Pmp47p and Ant1p [91]. PMP34 likely represents the mammalian ortholog of Ant1p, as it was able to partially complement the MCFA β-oxidation defect of the *ant1*Δ mutant, and showed some ATP exchange activity when reconstituted into liposomes [92]. However, due to the current lack of reports on the effects of PMP34 knock-down by RNAi or on PMP34 knock-out mice, its physiological role remains elusive.

A proteomic approach has led to the identification of a candidate peroxisomal adenine nucleotide translocator from *Arabidopsis*. PMP38 exhibits 25% and 26% amino acid identity to human PMP34 and *C. boidinii* PMP47, respectively and was shown by a combination of cell fractionation and immunocytochemical analysis to be an integral peroxisomal membrane protein [93]. Although the pattern of developmental changes in PMP38 abundance strongly suggest involvement in β-oxidation, the *in vivo* function has yet to be determined, for example, by characterisation of knock-out mutants or antisense lines.

2.3. CoA and phosphate transport

Peroxisomal acyl-CoA synthetases such as yeast Faa2p as well as 3-ketoacyl-CoA thiolases require free CoA within peroxisomes. Although some CoA probably enters the peroxisomal matrix thioesterified with LCFA via the Pxa1p–Pxa2p transporter of yeast, stoichiometry suggests that a certain pool of CoA must be provided by other means to maintain MCFA activation and the flux through the β-oxidation spiral. Furthermore, activation and degradation of MCFA occur normally in *pxa1*Δ*pxa2*Δ mutant cells [23]. It is currently unclear whether peroxisomes contain a CoA transporter as has been suggested for mitochondria. Here, the MCF member Leu5p is required for the accumulation of CoA in the mitochondrial matrix and the related human Grave's disease protein can functionally replace Leu5p [94]. It is not entirely impossible that this carrier also localises to peroxisomes.

Intraperoxisomal ATP-dependent fatty acid activation generates pyrophosphate that probably decomposes into two molecules of orthophosphate, which have to leave the peroxisome for regeneration of ATP. Evidence for a phosphate transporter was recently provided using proteoliposomes reconstituted from peroxisomal membranes that had been purified from bovine kidney [95]. Although the molecular identity of this transporter remains to be determined, it differed from the mitochondrial transporter in terms of sensitivity towards N-ethylmaleimide (NEM) and p-hydroxyphenylglyoxal (PHGP), as only the mitochondrial activity was adversely affected by these compounds [95].

2.4. Import of cofactors and regeneration of reducing equivalents

Genetic studies and transport assays utilising isolated peroxisomes suggest that the peroxisomal membrane is not freely permeable to bulky cofactors, implying that the peroxisome has separate pools of NAD^+, $NADP^+$, and FAD [9,10]. Acyl-CoA oxidase absolutely requires FAD, but the holoenzyme is assembled in the cytosol and is imported into peroxisomes as a folded protein [96]. Alcohol oxidase from methylotrophic yeasts also binds its cofactor in the cytosol, although in this case the assembly into an active octamer occurs inside peroxisomes [97,98]. Thus it appears likely that peroxisomal FAD-containing oxidases generally acquire their cofactor in the cytosol, and a peroxisomal FAD transporter might not be required. Peroxisomal FAD is reoxidised with the concomitant generation of H_2O_2 which in turn is removed by catalase. This situation contrasts with the import of FAD into mitochondria: the mitochondrial inner membrane has at least two specific FAD transporters, with Flx1p representing a FAD exporter [99], but in this organelle proteins traverse the membrane in an unfolded state which necessitates an intramitochondrial cofactor charging of the apo-flavoenzymes.

NADH is produced in the course of β-oxidation by the dehydrogenase reaction of the multifunctional enzyme and must be regenerated via one or more redox shuttles analogous to those in mitochondria. In mammals, pyruvate or dihydroxyacetone phosphate are likely to serve as the electron acceptors, through the action of lactate dehydrogenase [100,101] and glycerol-3-phosphate dehydrogenase [102], respectively. In contrast, in yeast [10] and plants (Pracharoenwattana and Smith, personal communication), the electrons are transferred to oxaloacetate, which is converted to malate by peroxisomal malate dehydrogenase. This could operate in the context of two different shuttles: a malate-oxaloacetate shuttle, or a malate-aspartate shuttle in which reducing equivalents are transferred from peroxisomes to mitochondria [10,103]. The former is a simpler scenario in which malate is exported to the cytoplasm, where it is re-oxidised by cytosolic malate dehydrogenase to oxaloacetate, which re-enters the peroxisome (Fig. 2A). NADH produced by cytosolic malate dehydrogenase is then re-oxidised by mitochondrial NADH dehydrogenases. The alternative malate-aspartate shuttle was originally proposed due to the fact that the inner mitochondrial membrane is relatively impermeable to oxaloacetate. In this case, oxaloacetate is not imported into the peroxisome, but rather is generated from aspartate and 2-oxoglutarate with the concomitant formation of glutamate in a transamination reaction catalysed by aspartate amino transferase [103,104]. This requires exchange of malate/2-oxoglutarate and glutamate/aspartate across the peroxisomal and mitochondrial membranes (Fig. 2B). However, this scheme has been contested by biochemical studies with leaf peroxisomes [105] and disruption of the *AAT2* gene of yeast does not

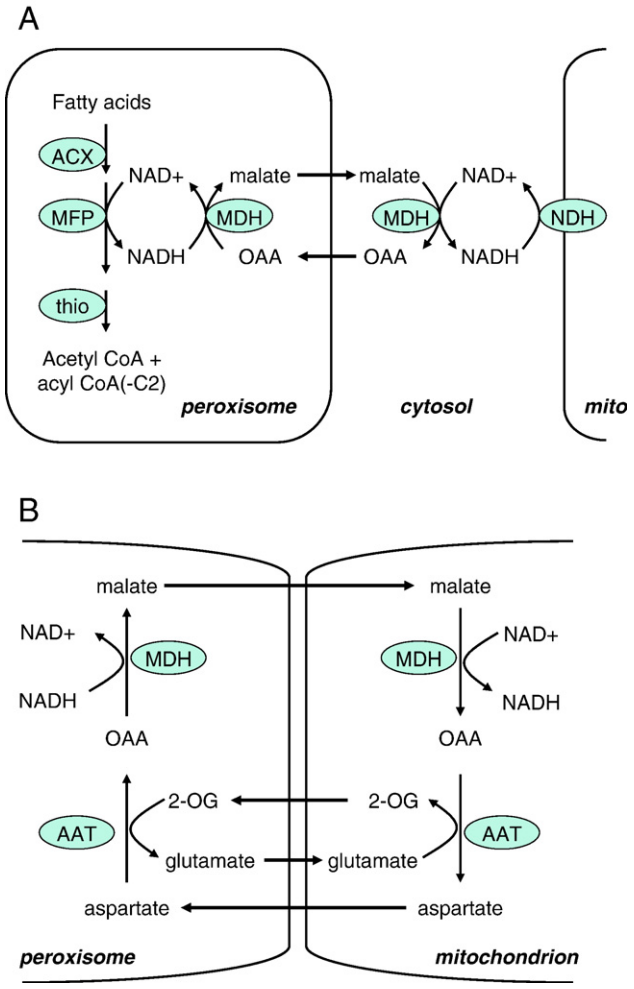

Fig. 2. Transport of reducing equivalents. The dehydrogenase reaction of the multifunctional protein of β-oxidation requires NAD^+. Since the peroxisomal membrane is impermeable to nucleotides and nucleotide-containing cofactors, two redox shuttles involved in regeneration of peroxisomal NAD^+ have been proposed for plants and yeast. Both systems require exchange of organic anions across the peroxisomal membrane, which may be executed by specific carriers or porin-like proteins. (A) The malate-oxaloacetate shuttle. Peroxisomal malate dehydrogenase (MDH) converts oxaloacetate (OAA) to malate with the concomitant oxidation of NADH. Malate is exported to the cytosol, where it is re-converted to OAA by the action of cytosolic MDH. NADH produced in this reaction is re-oxidised by mitochondrial NADH dehydrogenases (NDH). Import of OAA into the peroxisome completes the shuttle. Abbreviations: ACX, acyl-CoA oxidase; MFP, multifunctional protein; thio, thiolase. (B) The malate-2-oxoglutarate shuttle. As in the malate-oxaloacetate shuttle, malate produced from OAA by peroxisomal MDH is exported from the peroxisome. Malate is then transported into mitochondria where it is converted to OAA by MDH. OAA then undergoes conversion to aspartate by aspartate amino transferase (AAT), with the concomitant formation of 2-oxoglutarate (2-OG) from glutamate. 2-OG is transferred from mitochondria to peroxisomes where the reverse reaction occurs, catalysed by peroxisomal aspartate amino transferase, to yield OAA. This shuttle also requires the transport of aspartate and glutamate between mitochondria, peroxisomes and cytosol. Redrawn from Elgersma and Tabak [154].

affect growth on oleate, indicating that it is not essential for the regeneration of intraperoxisomal NAD^+ [106].

Regardless of the specific redox shuttle involved in regeneration, in all cases, NAD(H) must move back and forth from the multifunctional enzyme to a dehydrogenase and is therefore only transiently bound to the enzymes. It might be that NAD^+ is imported in the manner of FAD through cytosolic association and subsequent joint transit with NAD^+-dependent enzymes. Whether such a mechanism would be sufficient to establish the peroxisomal pool of NAD(H) remains unclear. It appears more likely that additional, low efficiency routes for provision of peroxisomal NAD^+ exist.

Similar arguments account for the supply of peroxisomes with $NADP^+$. This cofactor is required for the β-oxidation auxiliary enzyme Δ^2-Δ^4-dienoyl-CoA reductase, which is essential for the degradation of unsaturated fatty acids with double bonds at even-numbered positions [107]. NADPH is regenerated inside peroxisomes via an isocitrate/2-oxoglutarate shuttle [108–110], similar to that which links the mitochondrial and cytosolic pools of NADPH in rat liver (Fig. 3). Oxidation of isocitrate is catalysed by the peroxisomal $NADP^+$-dependent isocitrate dehydrogenase, Idp3p. This reaction regenerates NADPH and yields 2-oxoglutarate which is exported from the peroxisome and is possibly re-reduced to isocitrate by the cytosolic isocitrate dehydrogenase, Idp2p. The reverse reductive carboxylation of 2-oxoglutarate appears feasible under physiological conditions, since the K_m values of Idp2p are similar for isocitrate and 2-oxoglutarate, albeit V_{max} is about 20 times lower for the latter substrate [111]. The shuttle is thought to be completed by the import of isocitrate into the peroxisome [109,110], but the carriers required for shuttling of isocitrate and 2-oxoglutarate remain elusive. An alternative route for the peroxisomal regeneration of NADPH involves glucose-6-phosphate dehydrogenase [112].

The operation of an isocitrate/2-oxoglutarate shuttle or a glucose-6-phosphate dehydrogenase-dependent mechanism for NADPH regeneration in plant peroxisomes still requires experimental confirmation, although genes encoding a number of the requisite components have been identified at least *in silico* [104]. However, the identity of the dicarboxylate carriers which function in peroxisomal redox shuttles remains obscure. Mitochondria contain several dicarboxylate carriers which might co-target to peroxisomes [86]. Small amounts of specific carriers might have escaped detection in the various proteomic approaches aimed at identifying peroxisomal membrane proteins due to the prevalent problem of mitochondrial contamination [113,114]. Alternatively, peroxisomal porins which have not yet been identified at the molecular level could be responsible for transport of shuttle metabolites (see Antonenkov and Hiltunen, this issue).

2.5. Nudix hydrolases and regulation of cofactor and co substrate concentration

In addition to import and regeneration, export must be considered as a factor affecting the intraperoxisomal pools of NAD(P)H and CoA. Nudix hydrolases comprise a diverse family of enzymes which hydrolyse a diphosphate linkage in a variety of nucleotide-containing compounds and appear to play an important role in the regulation of intraperoxisomal cofactor concentration and possibly removal of damaged cofactors [115]. Peroxisomal representatives identified to date include

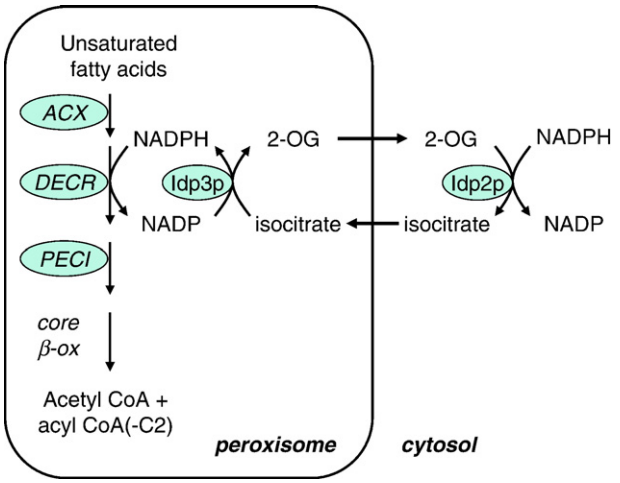

Fig. 3. The isocitrate-2-oxoglutarate shuttle. In addition to the chain-shortening β-oxidation system, the oxidation of unsaturated fatty acids requires auxiliary enzymes for the elimination of double bonds, including Δ^2-Δ^4-dienoyl-CoA reductase (DECR) and Δ^3-Δ^2-enoyl-CoA isomerase which may be provided by multifunctional protein (MFP) or by a monofunctional peroxisomal Δ^3-Δ^2-enoyl-CoA isomerase (PECI). The requirement of DECR for NADPH requires a system for the regeneration of peroxisomal NADPH. The isocitrate-2-oxoglutarate shuttle which employs peroxisomal (Idp3p) and cytosolic (Idp2p) isocitrate dehydrogenase has been proposed to occur in yeast and may also occur in plants. The mechanism of isocitrate and 2-oxo-glutarate transport across the peroxisomal membrane is unknown, but may involve specific carriers or porins which have been shown to transport dicarboxylic acids. Abbreviations: ACX, acyl-CoA oxidase; 2-OG, 2-oxoglutarate. Modified from van Roermund et al. [110].

NAD(P)H diphosphatases which exhibit substrate preference for reduced dinucleotides [116,117] and CoA diphosphatases, which handle CoA, CoA esters and oxidised CoA with differing efficiency [118–121]. Importantly from a transport perspective, these enzymes have the net effect of degrading bulky cofactors to smaller molecules which can readily exit the peroxisome.

3. Export of acetyl- and acyl-units

The export of acetyl-CoAs generated by peroxisomal β-oxidation is accomplished by either of two systems, a carnitine shuttle or through the export of citrate. This is because acetyl-CoA is not able to readily traverse the peroxisomal membrane, whereas the compounds produced are. The relative importance of the two modes varies between species. Genetic evidence indicates that both systems operate in yeast [10], whereas in plants only citrate export occurs [122]. Mammals lack the glyoxylate cycle and a peroxisomal citrate synthase does not exist, suggesting that here the carnitine-mediated export is the exclusive mode of acetyl-removal. An alternative route, which predominates in some mammalian tissues, is the export of acetate generated from acetyl-CoA by the action of peroxisomal thioesterases [1].

3.1. The carnitine shuttle

The existence of a carnitine shuttle is supported by the localisation of yeast carnitine acetyl transferase Cat2p, which is found in both mitochondria and peroxisomes [123]. In peroxisomes, the enzyme is thought to catalyse transfer of the acetyl moiety from CoA to carnitine, whereas in mitochondria it would catalyse the reverse reaction thereby enabling the condensation of acetyl-CoA with oxaloacetate to form citrate. As a consequence, acetyl-carnitine has to traverse both the peroxisomal as well as the inner mitochondrial membrane. Mitochondrial transport is accomplished by the carnitine carrier Crc1p, a member of the mitochondrial carrier family, which transports acetyl-(and propionyl-) carnitine in exchange for carnitine [124,125]. The important role the *CAT2* and *CRC1* genes play in fatty acid utilisation is also indicated by the induction of their expression in the presence of oleate [126]. However, and in contrast to the dually localised Cat2p transferase, the carnitine carrier is apparently absent from the yeast peroxisomal membrane [125]. This is also true for the *Aspergillus nidulans* carnitine carrier AcuH [127,128]. Which peroxisomal protein therefore enables the exit of acetyl-carnitine and the import of carnitine remains elusive.

In mammals, carnitine octanoyl transferase with a substrate preference for MCFAs additionally resides within peroxisomes [1,129,130]. This enzyme is required because peroxisomal β-oxidation usually does not go to completion. Rather, the chain-shortened substrates are transported to mitochondria as carnitine conjugates, where they are reconverted into the CoA esters and further degraded by the resident β-oxidation system. The broader substrate specificity of the mitochondrial carnitine acylcarnitine carrier CAC, which also accepts longer carnitine fatty acyl moieties, reflects the distinct distribution of β-oxidation systems in mammals and yeast [131]. A cluster of three carnitine transporters has been identified in the mouse genome with distinct subcellular localisation and affinities for carnitine. Recently, it has been suggested that *OCTN3* encodes a peroxisomal medium-affinity carnitine transporter [132,133]. Fusing OCTN3 to green fluorescent protein (GFP) and immunogold labelling using anti-OCTN3 antibodies revealed that this isoform of the family might indeed be a predominantly peroxisomal protein. The antibodies also reacted with a protein in human fibroblasts that was absent in a *pex19* patient cell line, in which the steady state concentration of several PMPs is below the detection limit due to a specific defect in peroxisome membrane biogenesis [134]. In light of the fact that the OCTN transporters belong to the organic cation transporter family that is not related to the MCF, the relevance of a reported cross-reactivity of an antibody against the mitochondrial carnitine carrier (CAC) with a protein from purified rat peroxisomes remains unclear [135].

Intriguingly, a carnitine acyl carrier homologue, BOU, has also been identified in *Arabidopsis* mitochondria. *bou* mutant plants exhibit a striking phenotype in which growth is arrested after germination but lipids are still degraded [136]. However, the function of this transporter in relation to peroxisomal metabolism remains obscure, since higher plants possess neither peroxisomal carnitine transferases nor a mitochondrial β-oxidation system.

3.2. Carnitine-independent export from peroxisomes: the role of thioesterases

In mammals, a carnitine-independent route for exporting acyl units from peroxisomes has been demonstrated [1,137]. Here, CoA esters are hydrolysed within peroxisomes to yield free acids which are transported to mitochondria and re-activated to their respective CoA esters for completion of β-oxidation. Hydrolysis of CoA esters is catalysed by peroxisomal thioesterases which vary in substrate specificity [138].

Interestingly, the *Arabidopsis* genome contains several candidate peroxisomal thioesterases [104,139], one of which (ACH2) has already been characterised [140]. In addition to roles in fatty acid β-oxidation, these may play an important role in the export of diverse, plant-specific metabolites synthesised in the peroxisome. For example, the final biosynthetic steps of the carboxylate-containing plant hormones, jasmonic acid and indole acetic acid, occur in peroxisomes. Following one (IBA to IAA) or three (OPDA to jasmonic acid) complete rounds of peroxisomal β-oxidation, the hormones are thought to leave the organelle as free acids, particularly in light of the absence of peroxisomal carnitine transferases. This would require a thioesterase-catalysed release from the CoA ester. Additional roles proposed for peroxisomal thioesterases include the regulation of acyl-CoA/free CoA levels and the prevention of CoA sequestration by intermediates which are β-oxidised inefficiently [138].

3.3. Diversion of acetyl-CoA through citrate synthase; transport requirements of the glyoxylate cycle

Citrate synthase catalyses the formation of citrate from oxaloacetate and acetyl-CoA with the liberation of free CoA. Yeast peroxisomal citrate synthase Cit2p also participates in the glyoxylate cycle, but is not essential for growth on nonfermentable carbon sources since the mitochondrial TCA cycle isoforms Cit1p and/or Cit3p can substitute for Cit2p (for more details, see Kunze et al., this issue). Acetyl-CoA generated within peroxisomes during β-oxidation also does not accumulate in a *cit2* deletion because of the redundant carnitine

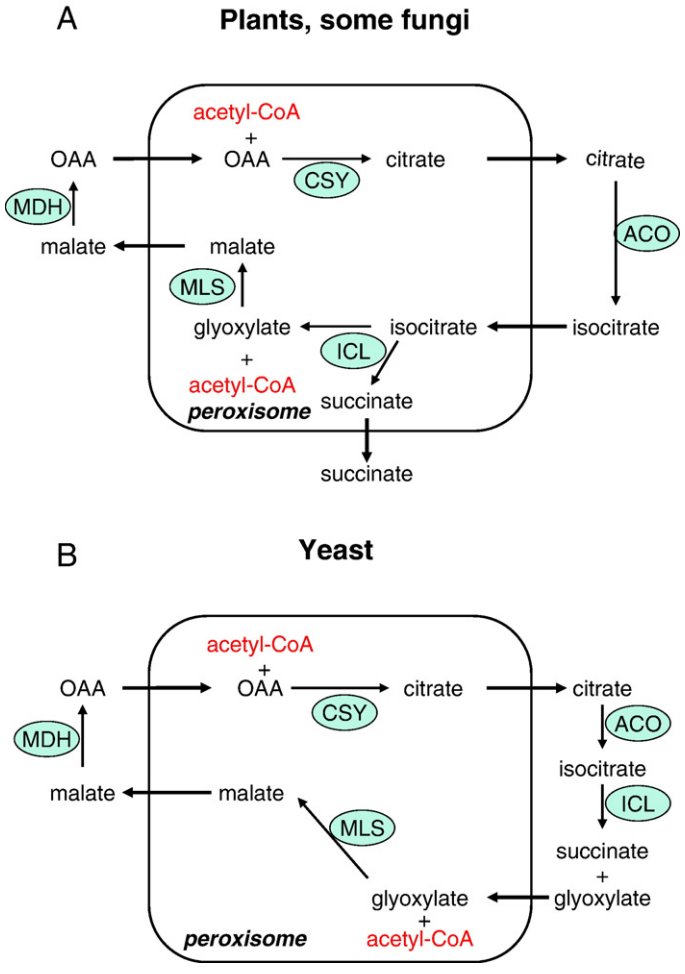

Fig. 4. Transport requirements of the glyoxylate cycle. The enzymes of the glyoxylate cycle are located in both peroxisomes and cytosol and isocitrate lyase is differentially localised in plants and fungi. This requires the transport of several different metabolites across the peroxisomal membrane but the precise biochemical nature of the transport steps in the glyoxylate cycle is unknown. Note that this scheme requires cytosolic malate dehydrogenase (MDH): peroxisomal MDH does not participate in the glyoxylate cycle, but rather operates in the reverse direction, in the regeneration of reducing equivalents shown in Fig. 2. The export of malate and import of oxaloacetate (OAA) correspond to the malate-OAA shuttle (Fig. 2A). Abbreviations: CSY, citrate synthase; ACO, aconitase; ICL, isocitrate lyase; MLS, malate synthase.

conjugation system described above. However, by deleting both *CIT2* and *CAT2* or *CRC1*, growth on oleate is abolished, because now acetyl-CoA is trapped within peroxisomes.

In *Arabidopsis*, two glyoxysomal citrate synthases Csy2 and Csy3 fulfil an analogous function in β-oxidation, with the difference that a carnitine bypass does not operate [122]. The double *csy2, csy3* mutant therefore exhibits an extremely severe phenotype: like *cts* and other strong β-oxidation alleles [31,141], *csy2, csy3* seeds are dormant and will only germinate when the seed coat is removed and sucrose is supplied. Moreover, rescued seedlings do not develop further when transferred to soil, indicating the importance of citrate export in plant growth and development [122].

Citrate produced by peroxisomal metabolism is exported to the cytosol and ultimately enters the mitochondrial TCA cycle, but also participates in the glyoxylate cycle to provide C4 biosynthetic precursors. The operation of the glyoxylate cycle requires the transport of intermediates across the peroxisomal membrane since enzymes of the glyoxylate cycle are located both inside and outside the peroxisome (Kunze et al., this issue). The differential localisation of enzymes in plants and fungi results in different transport requirements for the glyoxylate cycle: export of citrate and malate and import of oxaloacetate are common to both plants and fungi, but organisms in which isocitrate lyase is cytosolic must import glyoxylate whereas organisms with peroxisomal isocitrate lyase require the import of isocitrate and export of succinate (Fig. 4). The transport requirements of the glyoxylate cycle are considered in detail by Kunze et al. (this issue).

4. Intraperoxisomal pH

The existence of a proton gradient across the peroxisomal membrane is a long-standing matter of debate and relates to the question as to whether peroxisomes are impermeable to small molecules *in vivo*. Arguments in favour of a peroxisomal pore with an exclusion size of about 300 Da are presented by Antonenkov and Hiltunen (this issue). Data supporting the closed-compartment hypothesis emerged from analyses that were concerned with the pH of peroxisomes. Early evidence for a proton gradient across the peroxisomal membrane was obtained from studies with yeast. Upon induction of *Hansenula polymorpha* peroxisomes with methanol, a second phosphate pool became discernible through ^{31}P NMR studies measuring the pH-dependent chemical shifts of phosphate: in addition to the cytosolic phosphate peak, a peroxisomal peak attributable to a lower pH was observed [142]. Furthermore, the weak base DAMP, which accumulates specifically in acidic compartments, was detectable in peroxisomes of intact spheroplasts by immunocytochemistry [143]. Dissipation of intracellular pH gradients by chloroquine resulted in the disappearance of the second phosphate peak and DAMP was no longer located in peroxisomes, consistent with the existence of an endogenous ΔpH across the peroxisomal membrane. At that time, the generation of a proton gradient was explained by a (hypothetical) peroxisomal proton-translocating ATPase [144,145], but the molecular identity of this activity has remained elusive.

More recent *in vivo* measurements of the peroxisomal pH have caused considerable confusion. Determination of the peroxisomal pH in human fibroblasts with a membrane-permeable peptide harbouring a C-terminal peroxisomal targeting signal type 1 (PTS1) and labelled with the pH-sensitive fluorescent moiety carboxyseminaphtofluorescein (SNAFL-2) confirmed the existence of a pH gradient, but apparently with a reversed orientation, i.e. basic inside, with a peroxisomal pH of 8.2 [146]. The gradient could be dissipated by the addition of uncoupler and was apparently absent in a cell line harbouring a mutated *PEX7*, which exhibits a specific import defect of proteins with a PTS2. The approach used relies on the pH-dependent emission ratio of the fluorophore at two different wavelengths and is principally well suited for such measurements. A limitation of the method might arise from the low intracellular concentration of peptides dispersed throughout the cytosol, making cytosolic pH measurements troublesome. Its general applicability is further restricted by the possible extrusion of these peptides via multiple drug resistance (MDR) transporters [147] and the uptake of such peptides via endocytosis in yeast (our unpublished observation).

Variants of GFP allow a similar detection of pH-dependent emission intensities with the advantage of intracellular protein expression. Expression of one of these variants, the so-called ratiometric pHluorin [148] as an SKL-tagged variant in Chinese hamster ovary (CHO) and human fibroblasts revealed that the peroxisomal pH adapts to that of the cytosol [149]. As a consequence, the compartment was classified as being proton-permeable, not taking into account that changing a gradient on one side evokes an adjustment on the other side of the membrane. In apparent contrast, by expressing the same protein in yeast, a pH value more acidic than that of the cytosol was determined for peroxisomes [88]. Similar emission ratios of ratiometric pHluorin were observed for cytosolic and peroxisomal variants in both systems, but attempts to calibrate the system from outside the cell by Jankowski et al. [149] led to a different interpretation of the results.

Yellow fluorescent protein (YFP) and a point-mutated version with an altered pKa value were also used to determine the pH of yeast peroxisomes. Here, the absolute fluorescence intensity (rather than a ratio of intensities) was correlated with the pH, which possibly makes the system more vulnerable to differences in expression and distribution of the fluorescent protein. The outcome of this study was an alkaline peroxisomal pH that was even higher in several mutants affected in β-oxidation, whereas in *ant1*Δ cells, a pH gradient was not observed. A hypothesis was put forward that the imposed pH gradient serves as a driving force for the passive transport of free MCFAs, which would deprotonate more readily inside peroxisomes in a basic milieu [89]. However, this idea is hard to reconcile with the observed depletion of the gradient in the absence of Ant1p, which increases the lumenal proton concentration, unless an intraperoxisomal ATP-consuming reaction or transport process overcompensates this effect. Support for a basic pH also enters from studies with *Penicillium chrysogenum* suggesting a slightly alkaline pH for the microbody lumen in this fungus [150]. Finally, the basic pI of

several peroxisomal matrix enzymes has been invoked to support an argument for an alkaline peroxisomal pH [146].

The jury is out there to track down possible caveats in the various methods applied, which should lead to the desirable clarification of this issue. An important factor to be considered in future studies is the influence of growth conditions, developmental state and cell type on the peroxisomal pH. For instance, methanol utilisation in *H. polymorpha* requires an acidic peroxisomal pH, since the substrate specificity of dihydroxyacetone synthase requires formaldehyde in its protonated form [151]. What seems clear is that in yeast, Ant1p contributes to the formation of a pH gradient as a direct consequence of its proton-compensated transport mode [88]. Although the existence of an H^+ATPase is unlikely, additional factors might contribute to the maintenance of this gradient. To date it can only be speculated whether a peroxisomal proton gradient is needed to drive other transport processes, though physicochemical considerations predict that the existence of a pH gradient between the cytosol and peroxisomal lumen is likely to be a key component in determining the extent of passive transport of free acids across the peroxisomal membrane.

5. Conclusions and perspectives

Although considerable progress in understanding of peroxisomal transport processes has been made in recent years, many questions remain to be resolved. Several novel peroxisomal transporters have been reported recently, including a Ca^{2+} ATPase in *Drosophila* [152] and a peroxisomal protein from brome grass (*Bromus inermis)* which is similar to amino acid-selective channel proteins from the chloroplast outer membrane [153]. Nevertheless, knowledge of peroxisomal metabolism predicts the existence of further metabolite transporters involved in organism-specific peroxisomal processes, in addition to those mentioned elsewhere in this review. Identification of such transporters may come from several approaches, including proteomic analysis of peroxisomal membrane proteins, forward genetics approaches and characterisation of candidate genes using knock-down systems. A further, yet exciting, challenge is the rigorous characterisation of known transporters which will require the development of transport assays with isolated organelles and reconstituted, purified proteins. The past few decades have been an exciting era for peroxisome research and an improved understanding of peroxisomal transporters will undoubtedly enhance and extend our appreciation of the functions of this multipurpose organelle.

Acknowledgements

Work on peroxisomal ABC transporters in FLT's laboratory is supported by Biology and Biotechnology Research Council (BBSRC) grant P19769. Rothamsted Research receives grant-aided support from the BBSRC of the UK. The work of HR's laboratory is supported by the Deutsche Forschungsgemeinschaft grants SFB480 and RO2494/1.

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