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Review

Transfer of metabolites across the peroxisomal membrane ☆

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ABSTRACT

Peroxisomes perform a large variety of metabolic functions that require a constant flow of metabolites across the membranes of these organelles. Over the last few years it has become clear that the transport machinery of the peroxisomal membrane is a unique biological entity since it includes nonselective channels conducting small solutes side by side with transporters for 'bulky' solutes such as ATP. Electrophysiological experiments revealed several channel-forming activities in preparations of plant, mammalian, and yeast peroxisomes and in glycosomes of *Trypanosoma brucei*. The properties of the first discovered peroxisomal membrane channel – mammalian Pxmp2 protein – have also been characterized. The channels are apparently involved in the formation of peroxisomal shuttle systems and in the transmembrane transfer of various water-soluble metabolites including products of peroxisomal β-oxidation. These products are processed by a large set of peroxisomal enzymes including carnitine acyltransferases, enzymes involved in the synthesis of ketone bodies, thioesterases, and others. This review discusses recent data pertaining to solute permeability and metabolite transport systems in peroxisomal membranes and also addresses mechanisms responsible for the transfer of ATP and cofactors such as an ATP transporter and nudix hydrolases. This article is part of a Special Issue entitled: Metabolic Functions and Biogenesis of Peroxisomes in Health and Disease.

1. Introduction

Many different chemical compounds participate in cellular metabolism and most are classified as either hydrophobic or hydrophilic based on their solubility in water. Hydrophilic molecules require an aqueous environment for their synthesis and degradation. In contrast, the same environment is inappropriate for the conversion of hydrophobic compounds due to their low solubility in aqueous solutions. Eukaryotic cells, however, are perfectly equipped to deal with hydrophilic and hydrophobic molecules. These cells contain spaces filled with water, such as cytoplasm, mitochondrial and nuclear matrixes side by side with lipid bodies and different membrane systems accommodating hydrophobic molecules within lipid bilayers. However, a relatively small group of metabolites cannot be considered truly hydrophobic or hydrophilic. These metabolites are mainly amphipathic molecules containing both, hydrophilic and hydrophobic, portions in their structure. The most obvious examples are long- and very long-chain fatty acids which are poorly soluble in water at physiological conditions and tend to damage biological membranes owing to the detergent-like effect. In addition to fatty acids, some other amphipathic compounds such as precursors of bile acids, fatty alcohols, and hydrophobic amino acids belong to this family. Moreover, it includes not only amphipathic molecules but also other metabolites that are poorly soluble in water and in non-polar solvents such as purines (hypoxanthine, xanthine, and uric acid) and calcium salt of oxalate. An inability to sequester these compounds in water- or lipid-containing compartments seems to cause problems for cellular metabolism as exemplified by wide-spread human disorders as gout, kidney and gallbladder stones, and others. To prevent the accumulation of these compounds, eukaryotic cells mainly rely on a special set of organelles known as peroxisomes. Many peroxisomal enzymatic processes lead to increase in water solubility of the generated compounds relative to the initial substrates allowing further metabolism or excretion of the resulting products. Despite

Abbreviations: ABC transporters, ATP-binding cassette transporters; alkyl-DHAP synthase, alkyl-dihydroxyacetonephosphate synthase; BAAT, bile acid-CoA:amino acid N-acyltransferase; DHAP, dihydroxyacetonephosphate; DHAPAT, acyl-CoA:dihydroxyacetonephosphate acyltransferase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; LCFA, long-chain fatty acids; NMN, nicotinamide mononucleotide; OCTN, organic cation/carnitine transporter; ROS, reactive oxygen species; VLCAS, very long-chain acyl-CoA synthase; VLCFA, very long-chain fatty acids

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the profound diversity in metabolic functions of peroxisomes from different sources, these organelles share several common hallmark features such as morphology, biogenesis and, as has been revealed over the last few years, transmembrane transport system (see Table 1). The peroxisomal transmembrane transport system is the topic of this review, which follows previous contributions in the field by us [1,2] and others [3–5].

2. Peroxisomal membrane permeability to solutes

Mammalian peroxisomes are classified as members of the microbody family of organelles that includes also yeast and plant peroxisomes, glyoxysomes from plant seeds, glycosomes from unicellular eukaryotes of the Kinetoplastea order such as *Trypanosoma* and *Leishmania* species, and highly specialized structures from filamentous fungi — the so-called Woronin bodies. In this review we focus mainly on the metabolite transport in mammalian and yeast peroxisomes, and where it is appropriate, discuss important information about other members of the family.

Mammalian peroxisomes from liver and kidney are round or oval particles (0.5–1.0 μm in diameter) surrounded by a single membrane and containing a lumen filled with matrix proteins. Peroxisomes from other tissues (microperoxisomes) are smaller (0.1–0.4 μm) but show the same overall morphology. The lipid composition of the mammalian peroxisomal membrane is similar to the membrane of smooth endoplasmic reticulum [6]. The membrane contains no 'specific' phospholipids like cardiolipins or sphingolipids. However, the peroxisomal membrane phospholipids, especially phosphatidylcholine, are highly enriched with polyunsaturated arachidonic acid [7]. Several proteomic analyses of highly purified mammalian [8] and yeast [9,10] peroxisomes were unable to detect a high diversity of peroxisomal membrane proteins. As a rule, not more than 10 specific membrane proteins were recovered including several that are involved in peroxisomal biogenesis [8–10], see also Ref. [11–13]. Based on these observations one can predict rather simple machinery for transmembrane metabolite transfer in peroxisomes in comparison, for instance, to an inner mitochondrial membrane that contains more than fifty transporters specific for different solutes [14] side by side with several highly regulated ion channels [15]. The most abundant protein components in the membrane of peroxisomes isolated from rat or mouse liver are: three ABC half-transporters, the Pxmp2 protein known also as PMP22, and members of the Pex11 family of proteins [8,11–13].

With respect to solute transfer, two types of biomembranes have been described in living cells. The inner membranes of gram-negative bacteria, mitochondria, and chloroplasts, as well as plasma and lysosomal membranes are closed to solutes, i.e. they are unable to allow free transmembrane movement of inorganic ions and water-soluble metabolites. Instead, these membranes contain a large set of transporters specific for certain solute(s). In contrast, the outer membranes of gram-negative bacteria, mitochondria and chloroplasts are open to solutes because they contain proteins forming nonselective transmembrane channels (pore-forming proteins) that allow unrestricted diffusion of metabolites across the membrane. Hence, these membranes do not require specific transporters. It appears that the foregoing description does not fit well with our current knowledge of peroxisomal metabolite transfer. The controversy surrounding this issue lasted for more than 50 years starting from the first *in vitro* experiments on rat liver peroxisomes [16]. This study has revealed that contrary to e.g. lysosomal enzymes, the enzymes in peroxisomes do not show latency, i.e. they do not increase their activity after disruption of the organelle membrane by physical treatment or detergents. This was later confirmed by detection of the activity of peroxisomal enzymes (urate oxidase, L-α-hydroxyacid oxidase, and D-amino acid oxidase) in digitonin-permeabilized rat hepatocytes [17]. The results were interpreted as an indication that the peroxisomal membrane is open to solutes and contains pore-forming proteins. Some attempts have been made to describe the peroxisomal channel-forming activities using biochemical [18] and electrophysiological [19,20] approaches.

However, several lines of evidence were considered to be an indication that the peroxisomal membrane is closed to solutes and the results of some *in vitro* experiments (see earlier discussion) may be an artifact caused by disruption of the particles during isolation. Thus, latency of the peroxisomal enzyme acyl-CoA:dihydroacetonephosphate acyltransferase (DHAPAT) in digitonin-permeabilized fibroblasts was detected [21]. Disruption of the genes for the peroxisomal malate dehydrogenase, citrate synthase, and carnitine acetyltransferase, enzymes involved in metabolite shuttling in the yeast *Saccharomyces cerevisiae* was shown to block the β-oxidation of fatty acids in intact cells but not in cell lysates [22–24]. This indicates that as in mitochondria, the yeast peroxisomes require shuttle systems for transmembrane transfer of reducing equivalents and acetyl units. Therefore, it was concluded that the membrane of yeast peroxisomes is impermeable to NAD/H and acetyl-CoA under *in vivo* conditions. Furthermore, several groups established the presence of a pH gradient across the peroxisomal membrane [25–27] that was widely accepted as evidence that this membrane is closed to solutes (see later discussion for details). Finally, an ATP transporter, which belongs to the superfamily of mitochondrial inner membrane solute carriers, was detected in the membrane of peroxisomes from different sources [28–31].

In an attempt to resolve the issue of peroxisomal membrane permeability to solutes, we conducted *in vitro* experiments on highly purified peroxisomes isolated from rat liver [32,33]. The results revealed an unexpected property of the transport machinery of the peroxisomal membrane: it is able to discriminate between the sizes

Table 1
Metabolite transport proteins/activities of the peroxisomal membrane*.

Transport proteins/activities	Model organism	Mode of action	Substrate specificity	References
Pxmp2	Mouse	Non-selective channel	Small solutes	[59]
Channel-forming activity**	Mouse	Non-selective channels	Not known	[47]
–"–	Spinach leaf	–"–	Specific towards carboxylic acids	[44]
–"–	Castor bean	–"–	Not known	[45]
–"–	*Trypanosoma brucei*	–"–	–"–	(unpublished)
–"–	*Saccharomyces cerevisiae*	–"–	–"–	[48, 50]
Pex11	–"–	Not known	Medium-chain fatty acids (?)	[70]
ATP transporter				
SCL25A17	Human	Transporter (antipoter)	ATP/AMP	[29]
PNC1 and PNC2	*Arabidopsis thaliana*	–"–	–"–	[30,31]
Ant1p	*S. cerevisiae*	–"–	–"–	[28]
NAD^+ transporter (PXN)	*Arabidopsis thaliana*	–"–	NAD^+/AMP	[147]
Bile acids transport**	Bovine	Transporter	Bile acids	[127]
Transporters for small solutes**	Mammals	–"–	Carnitine, phosphate, and others	[79–81,107]

*The table does not contain information about peroxisomal ABC transporters. **Proteins responsible for the corresponding activities have not been described.

of the transported molecules allowing free transmembrane movement of solutes with molecular masses up to 300–400 Da but preventing diffusion of larger molecules. Therefore, one can consider the peroxisomal membrane as open to small solutes but closed for 'bulky' solutes. Remarkably, some 'bulky' solutes including ATP and cofactors such as NAD/H, NADP/H and CoA are essential for biological systems (Fig. 1A). As a whole, in terms of cellular physiology, our data indicate that the preroxisomal membrane is permeable to practically all cellular intermediate metabolites but severely restricts the diffusion of ATP and cofactors [1,32,33]. Interestingly, this concept allows us to reconcile most of the previously reported 'conflicting' data related to peroxisomal membrane permeability. In addition, it leads to several predictions important for analysis of peroxisomal functions. Thus, the peroxisomal membrane may contain channels to transfer small solutes as well as transporters specific for 'bulky' metabolites. As a result, under *in vivo* conditions, peroxisomes apparently possess their own pool of cofactors while they share a common pool of small metabolites with the cytoplasm (Fig. 1B).

3. Intraperoxisomal pH and Donnan equilibrium

At first glance, the existence of a pH gradient across any biological membrane is an obvious argument that proves this membrane is closed to solutes. Indeed, differences in concentrations of protons (H^+) and hydroxyl ions (OH^-) between the peroxisomal lumen and the surrounding cytosol may well be explained by the assumption that these small charged molecules do not pass freely across the peroxisomal membrane. Several groups have tried to detect the pH in the lumen of yeast [26,27,34] and mammalian [25,35] peroxisomes. As a whole, these studies produced conflicting results. *In situ* measurements on yeast cells demonstrated that the peroxisomal lumen of *S. cerevisiae* is alkaline [27] although the internal pH of peroxisomes in *Hansenula polymorpha* and *Candida utilis* is acidic [34]. Similarly, pH determination using the selective targeting of fluorochromes into peroxisomes of human fibroblasts revealed alkaline conditions in these organelles [25]. This was not confirmed by similar *in situ* experiments with the fluorescent protein pHluorin that showed near neutral pH in peroxisomes of CHO cells and high permeability of the peroxisomal membrane to protons [35]. Interestingly, attempts to analyze *in situ* the Ca^{+2} content in peroxisomes from mammalian cells also led to controversial results [36,37]. All these 'conflicting' data are not consistent with the concept that the peroxisomal membrane is closed to solutes. The membranes closed to solutes, e.g. inner mitochondrial or lysosomal membranes, contain energy-dependent gradient-forming ion pumps (H^+-ATPase) to sustain the corresponding transmembrane ion gradients. If the peroxisomal membrane is equipped with similar ion pumps, to explain differences in the intraperoxisomal pH, one would have to predict that the direction of gradients created by these pumps varies from species to species and even depends on cell type within the same organism. This conclusion is difficult to reconcile with the unidirectional, vectorial action of ion pumps and other transporters. Therefore, a mechanism other than an active transmembrane transport has to be considered to explain the formation of ion gradients in peroxisomes.

A mechanism known as Donnan equilibrium is well established in biological systems [38]. According to this mechanism, the formation of ion gradients require, instead of a membrane that is closed to solutes, a membrane that is open to any ion except charged macromolecules

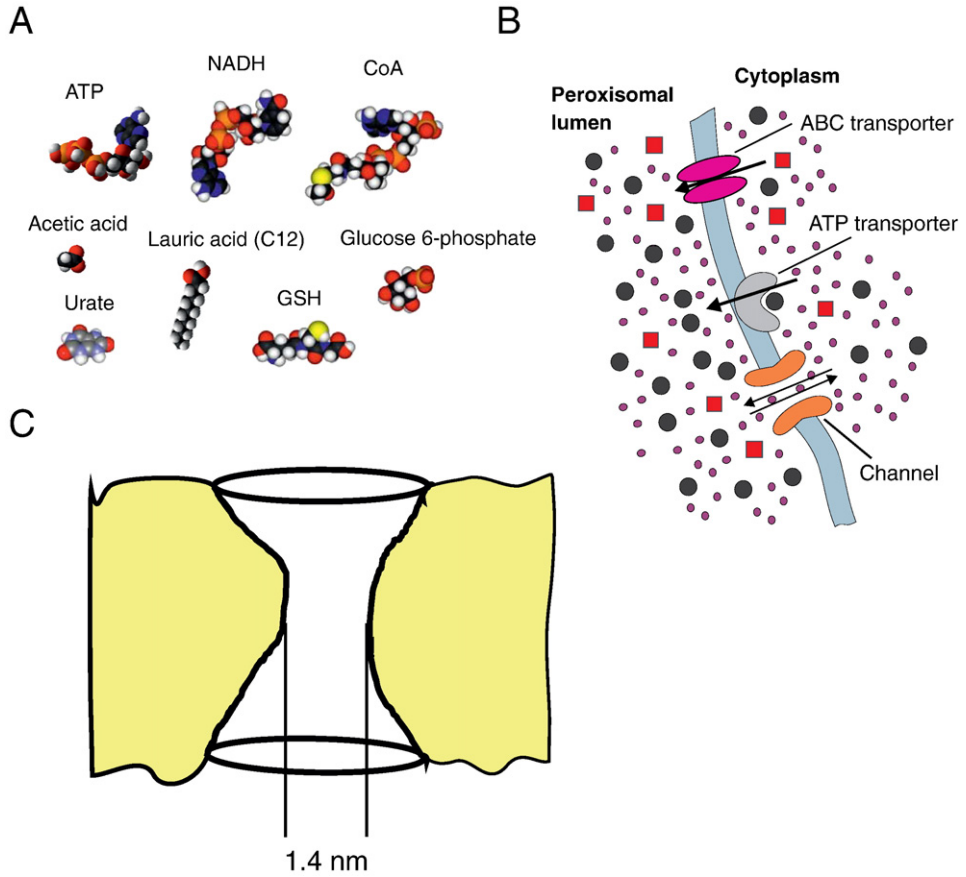

Fig. 1. Basic concept of the peroxisomal membrane permeability to metabolites.(A) Molecular models of 'bulky' (ATP, NADH, and CoA) and small solutes. (B) Role of selective transporters and non-selective channels in the transfer of 'bulky' (red squares and black circles) and small (red circles) solutes across the peroxisomal membrane. The transporters facilitate unidirectional transfer of bulky solutes (ATP transporter) and mainly hydrophobic compounds (ABC transporter) from the cytosol into peroxisomes leading to the creation of a separate pool of the corresponding metabolites in the peroxisomal lumen. In contrast, the channels allow transmembrane movement of small solutes by free diffusion. (C) Hypothetical shape of the Pxmp2 channel. The membrane bilayer is shown in yellow. Figures in panels A and C are at the same scale. Note that the size of the 'bulky' solutes, even without consideration of the hydrated radii of these compounds, is comparable with the diameter of the channel's pore.

such as proteins [38]. If the overall charge of impermeable macromolecules differ in two compartments separated by a membrane, the mobile ions that are able to cross the membrane freely, including protons, hydroxyl ions or Ca^{+2}, will preferably move according to their charge to one of the compartments to preserve electroneutrality. As a result, gradients of ions between two compartments are established. Thus, matrix proteins in peroxisomes from mouse or rat liver are mainly positively charged [2,39]. This indicates that the particles attract negatively charged solutes such as hydroxyl ions but repulse positively charged protons. Therefore, alkaline conditions can be expected in the lumen of peroxisomes from rodents. Remarkably, in peroxisomes not only protein macromolecules but also 'bulky' solutes such as negatively charged molecules of ATP are apparently involved in the formation of a Donnan equilibrium. This may be one of the reasons for the high variability of the intraperoxisomal pH.

In addition to peroxisomes the Donnan equilibrium apparently responsible for pH difference across the outer mitochondrial membrane [40]. This membrane is widely considered as open to solutes owing to the presence of voltage-dependent anion channel with size of the pore 2.7×2.4 nm [41].

4. Channel-forming activities of peroxisomal membrane proteins

Electrophysiological approaches using the reconstitution of proteins into artificial lipid bilayers or application of a patch-clamp technique to protein-containing giant liposomes are powerful tools to study channel-forming activities in membrane preparations from different cellular organelles. The methods are so sensitive that they allow detection and characterization of the permeability properties of even one channel molecule [42]. Nevertheless, the results of several initial attempts to use electrophysiological procedures to reveal the channel-forming activities in peroxisomal preparations from mammals [19,20] and yeasts [43] were inconclusive mainly due to the relatively low purity of the isolated fractions (see our previous review for more details [1]). In addition, these experiments were conducted using low concentrations of electrolyte (0.1–0.3 M KCl) at which, as has been shown later, the insertion of peroxisomal channel-forming proteins into an artificial membrane is quite ineffective. Measurements performed at higher electrolyte concentrations (usually 1.0 M KCl) readily detected the presence of channel-forming proteins with a single-channel conductance 0.33–0.35 nS in 1.0 M KCl in membrane preparations of spinach leaf peroxisomes [44] and glyoxisomes of castor bean endosperm [45]. The channels are strictly anion-selective with a ratio of Cl^- to K^+ permeability (P_{Cl}/P_K) of about 20. They are moderately voltage-dependent showing a tendency to close at holding potentials over ±40 mV. Interestingly, the single-channel conductance of these channels is not dependent linearly on bulk electrolyte concentration. This may indicate that not only the size of the channel's pore but also the charge of the selectivity filter (the narrowest part of the pore) determines the permeability properties of the channel. The channels from spinach leaf peroxisomes displayed selectivity towards organic anions such as malate, oxaloacetate, 2-oxoglutarate and other intermediates of photorespiration [44]. Therefore, they showed properties of so-called specific channels that have been initially found in the outer membrane of gram-negative bacteria [46].

To verify our predictions concerning peroxisomal membrane channels which are open to small solutes (see earlier discussion) we conducted a systemic search for channel-forming activities in highly purified preparations of peroxisomes from mouse liver [47], yeast *S. cerevisiae* [48], and glycosomes from the bloodstream form of *Trypanosoma brucei* (Gualdron-Lopez et al., unpublished results). The data obtained have led us to the overall conclusion that apparently all members of the microbody family contain channel-forming proteins in their membranes although the electrophysiological properties of these channels are quite different. Moreover, it seems that, with the exception of plant peroxisomes, the particles from the other groups of living species contain more than one type of channel-forming protein. Thus, electrophysiological monitoring of proteins solubilized from mouse liver peroxisomes revealed two types of channels with a conductance of 1.3 and 2.5 nS in 1.0 M KCl, respectively [47]. Both activities were resistant to voltage-dependent gating, which supports the conclusion that they belong to separate types of channels rather than represent two conformational states of the same channel. An overall peroxisomal channel-forming activity showed moderate cation selectivity with a K^+/Cl^- ratio close to 4.0. Importantly, comparative recording of the channel-forming activities in purified peroxisomal fractions and in the fractions containing mainly mitochondria but also fragments of endoplasmic reticulum and lysosomes revealed clear differences in the conductance patterns of the inserted channels. This indicates that the channel-forming activities registered in the peroxisomal fraction were not caused by an admixture of this fraction with other cellular organelles.

Detailed electrophysiological analysis has also been done on peroxisomal preparations from the yeast *S. cerevisiae* [48]. Initial observations revealed two abundant channel-forming activities with an average conductance of 0.2 and 0.6 nS in 1.0 M KCl, respectively. As in mammalian peroxisomes, the pattern of insertion events in the peroxisomal fraction from yeast cells was remarkably different from that of the mitochondrial fraction. The channel with the higher conductance showed only a limited preference to cations over anions (P_K^+/P_{Cl}^- ~1.3) and an unusual flickering at holding potentials over ±40 mV directed upward relative to its open state. It was stable in a fully open confirmation at a wide range of holding potentials (±100 mV). The properties of the channel with the lower conductance such as ion-selectivity, resistance to voltage-dependent gating and especially a frequent transition of current amplitudes to the level characteristic for the high-conductance channel all support the prediction that this high-conductance channel is a homodimer consisting of two low-conductance channels [48].

Perhaps, the most intriguing problem related to yeast peroxisomes is the reason for the bipartite distribution of glyoxylate cycle enzymes between these particles and the cytosol [49]. The cycle is instrumental in the conversion of the final product of peroxisomal β-oxidation – acetyl-CoA into succinate – a precursor for carbohydrate synthesis. It is catalyzed by five proteins including two peroxisomal acetyl-CoA-consuming enzymes: citrate synthase (Cit3p) and malate synthase (Mls1p). The other enzymes, isocitrate lyase (Icl1p), malate dehydrogenase (Mdh2p), and aconitase (Aco1p) are cytosolic constituents. Therefore, to sustain the whole cycle the intermediate metabolites such as citrate, malate, glyoxylate, and oxaloacetate must be transferred across the peroxisomal membrane [49]. Remarkably, the intracellular localization of glyoxylate cycle enzymes serves as a good indicator that the membrane of yeast peroxisomes is open to small solutes but restricts permeation of 'bulky' metabolites like acetyl-CoA or CoA. Indeed, the peroxisomal localization of acetyl-CoA-consuming enzymes allows them to use 'bulky' acetyl-CoA inside the particles. The reactions catalyzed by these enzymes result in the formation of small metabolites (malate and citrate), which freely penetrate the peroxisomal membrane. This may explain the 'useless' of the presence of Icl1p, Mdh2p, and Aco1p in peroxisomes since the lumen of these particles and the surrounding cytosol share a common pool of small solutes including metabolites of glyoxylate cycle. In line with this conclusion, our measurements on isolated yeast peroxisomes detected latency of cofactor-dependent enzymes. However, this phenomenon was not shown for aspartate aminotransferase that does not require for detection of its activity cofactors or other 'bulky' compounds [48]. Next, we tried to assess the involvement of peroxisomal channels in the transmembrane transfer of glyoxylate cycle intermediates using

an electrophysiological approach [50]. Unexpectedly, these experiments revealed the presence in the peroxisomal preparations of a high-conductance channel inducible by the reducing agent dithiothreitol together with previously described low-conductance channel-forming activities (see earlier discussion). The newly discovered channel with a conductance in a fully open state of 4.0 nS (1.0 M KCl) is moderately anion-selective ($P_{citrate}/P_K^+$ ~1.6) and does not show voltage-dependent gating at a wide range of holding potentials (±100 mV). This channel is able to transfer a broad spectrum of small solutes including metabolites of the glyoxylate cycle and apparently represents the main route connecting peroxisomal and cytoplasmic enzymes of this cycle.

Glycosomes are highly specialized members of the microbody family that were initially described in *T. brucei* [51]. This unicellular eukaryote belongs to the Kinetoplastea order which includes *Trypanosoma* and *Leishmania* species. Some of these organisms, including *T. brucei* are obligate parasites living in the mammalian bloodstream and transmitted by insects. The bloodstream form of *Trypanosoma* parasites relies totally on glucose from the host's blood as an energy source and uses glycolysis to metabolize carbohydrates with the formation of ATP by substrate-level phosphorylation. The first seven enzymes of the glycolytic pathway are localized in the glycosomes of *T. brucei* were they represent over 90% of the total protein content [51]. This is a unique location for glycolysis since in other organisms the pathway is cytosolic. How the metabolites of glycolysis are transported across glycosomal membrane is unclear. We proposed that the sieve properties of this membrane and membranes of mammalian and yeast peroxisomes are similar. All of them allow free transfer of small solutes but prevent movement of 'bulky' compounds including ATP and some cofactors. This prediction is in line with an experimentally validated hypothesis that the regulation of glycolysis in *Trypanosoma* species is achieved by separation of two ATP pools – cytosolic and glycosomal – by the glycosomal membrane [52,53]. Hence, this membrane, like the membrane of peroxisomes from mammals and yeasts, would be expected to contain pore-forming channels involved in the selective transfer of metabolites. Indeed, three main channel-forming activities with an average conductance of 7.5 nS, 2.0 nS and 1.0 nS (3.0 M KCl as an electrolyte) were detected in highly purified glycosomal preparations isolated from the bloodstream form of *T. brucei* (Gualdron-Lopez et al., unpublished work). All channels are resistant to gating in a range of voltages ±150 mV and show no subconductance transitions. The channel with a conductance of 2.0 nS is selective to anions (P_{Cl}^-/P_K^+ ~3.2) while the other two types of channels are slightly cation-selective (P_K^+/P_{Cl}^- ratios ~1.15 and 1.27 for high- and low-conductance channels, respectively). The conductance of the anion-selective channel is not dependent linearly on applied voltages, i.e. this channel shows current rectification that may indicate a functional asymmetry of the channel's pore and predicts an allosteric regulation of the channel properties.

5. Pxmp2 is a channel-forming protein

Pxmp2 is an abundant integral membrane protein in rodent liver peroxisomes [11,13]. The murine protein with a monomeric molecular mass of 22 kDa consists of 194 amino acids and contains four putative transmembrane domains. It is widely expressed in animal tissues although the expression rate is not affected by peroxisome proliferator clofibrate [13,54]. Pxmp2 belongs to a small family of membrane proteins consisting of four members in mammals: Pxmp2 itself, the *Mpv17* gene product [55], the Mpv17-like (MP-L) protein [56] and the *FKS24* gene product, which is still uncharacterized at the protein level. The yeast *S. cerevisiae* contains two proteins homologous to Pxmp2: Sym1p [57] and the *YOR292c* gene product. Interestingly, the protein homologous to Pxmp2 is a membrane component of Woronin bodies and participates in the biogenesis of these organelles [58].

Initial evidences indicating that Pxmp2 might be a pore-forming channel came from experiments which showed that a fraction of the peroxisomal membrane proteins from rat liver containing, among other components, the Pxmp2 protein was instrumental in promoting leakage of solutes such as radioactive sucrose from liposomes preloaded with these solutes [18]. On the basis of these and other experiments, the authors speculated that the peroxisomal membrane is permeable to cofactors of β-oxidation. Later this conclusion was widely challenged mainly due to the discovery of the ATP transporter (see Section 6) and the shuttle systems (see Section 7) in mammalian peroxisomes.

To study the function of Pxmp2 we have used two independent approaches: (1) electrophysiological detection of the channel-forming activity of the isolated protein and (2) deletion of the gene coding for Pxmp2 in mice in order to characterize the physiological importance of this protein *in vivo* [59]. Electrophysiological experiments revealed that the native Pxmp2 purified from peroxisomes of mouse liver is indeed a protein forming a general diffusion pore in the membrane. The isolated Pxmp2 is a homotrimer displaying a channel-forming activity mainly at three conductance levels: 0.45 nS, 0.9 nS, and 1.3 nS in 1.0 M KCl as an electrolyte. The channel at the high-conductance state (1.3 nS) is closed spontaneously at extreme holding potentials (±100 mV) showing three equal subconductance levels of 0.45 nS each. This and other observations suggest that each subunit of the Pxmp2 trimer forms a separate pore in the membrane. The channel is weakly cation-selective ($P_K^+/P_{Cl}^- \approx 2.3$) and usually stable in an open configuration for long periods of time (minutes). The estimated diameter of the channel is 1.4 nm, which is well over the dimensions of small solutes but comparable with the size of 'bulky' metabolites. Experiments with solutes and non-electrolytes of different sizes revealed that the Pxmp2 channel enables free diffusion across the membrane of compounds with molecular masses up to 300 Da. The permeation of larger molecules, from 300 Da to 500–600 Da in size, is partially restricted, while larger metabolites do not enter the channel (Fig. 1C). The channel-forming activity of Pxmp2 was confirmed with recombinant protein expressed in insect cells. Analysis of liver peroxisomal preparations showed that the channel-forming components with conductance 1.3 nS in 1.0 M KCl were lost in *Pxmp2*$^{-/-}$ mice. Moreover, a detailed biochemical investigation of Pxmp2-deleted mice revealed evidence for a partial restriction of peroxisomal membrane permeability to solutes *in vivo* and *in vitro* [59].

Surprisingly, the overall phenotype of Pxmp2-deficient mice is near normal. The only exception is the inability of female *Pxmp2*$^{-/-}$ mice to nurse their pups owing to an affected development of the mammary gland epithelium (Vapola M. et al., unpublished work). The mild phenotype of Pxmp2-deficent mice may be explained by functional redundancy of peroxisomal membrane channels. Indeed, deletion of Pxmp2 only led to a partial restriction in the permeability of isolated liver peroxisomes to uric acid. Likewise, substantial channel-forming activity still remained in the Pxmp2-deficient particles indicating that channels other than Pxmp2 may sustain the peroxisomal transmembrane transport [59]. The obvious candidates for the role of peroxisomal membrane channels are proteins homologous to Pxmp2 — Mpv17 and ML-P. Initial reports localized both these proteins to peroxisomes [56,60,61]. However, later they were found in the inner mitochondrial membrane [62,63]. The function of Mpv17 and ML-P in mitochondria is still unknown and attracts increasing attention mainly because deletion of Mpv17 in mice provokes symptoms of premature aging [64]. Our own experiments (unpublished observations) such as analytical subcellular fractionation of mouse liver homogenates followed by immunodetection of Mpv17 and creation of double – Mpv17$^{-/-}$/Pxmp2$^{-/-}$ – knock-out mice support the mitochondrial localization of Mpv17. Interestingly,

like Pxmp2, the purified recombinant Mpv17 protein shows channel-forming activity that can be easily registered and analyzed using electrophysiological approaches (Antonenkov VD, unpublished results).

Among yeast proteins homologous to Pxmp2, Sym1p was localized to the inner mitochondrial membrane [57]. Recently it was suggested that this protein may be involved in the mitochondrial transmembrane transport [65]. Until now, Yor292cp was considered to be a component of the vacuolar membrane [66]. However, our preliminary results indicate a peroxisomal localization, and the purified recombinant protein shows channel-forming activity.

Other potential candidates for the role of peroxisomal membrane channels are proteins of the Pex11 family. This family consists of three membrane-associated proteins in mammalian (Pex11α, Pex11β, and Pex11γ) and yeast (Pex11, Pex25, and Pex27) peroxisomes. The main focus of research on these proteins is their role in the biogenesis of peroxisomes [67–69]. However, at least one report suggests that yeast Pex11 protein has a role in peroxisomal transmembrane transport [70]. Deletion of the *Pex11* gene was accompanied by inhibition of the peroxisomal β-oxidation of medium-chain fatty acids but only if the membrane of the organelles was intact. Disruption of the membrane by detergents restored the β-oxidation rate to near normal levels. The β-oxidation of long-chain fatty acids was not affected. On the basis of these experiments the authors concluded that yeast Pex11 protein is somehow involved in the transportation of free medium-chain fatty acids and/or other metabolites of β-oxidation across the peroxisomal membrane. In contrast to the long- and very long-chain fatty acids which are highly hydrophobic and require specific transmembrane carriers such as ATB-binding cassette transporters (ABC transporters) to reach the peroxisomal lumen, the medium- and short-chain fatty acids are soluble in water and may well cross the membrane by diffusion through the channels.

6. Peroxisomal ATP transporter

An enzyme consuming ATP – very long-chain acyl-CoA synthase (VLCAS) – has been localized to mammalian peroxisomes, more precisely, to the inner surface of the peroxisomal membrane [71,72]. The same localization has been described for long-chain acyl-CoA synthase (Faa2p) from the yeast *S. cerevisiae* [3,73]. The mammalian enzyme is apparently involved in the activation of pristanic acid — the final product of the peroxisomal α-oxidation of phytanic acid [74] whereas the yeast synthase is responsible for the activation of medium-chain fatty acids [73]. The activated fatty acids enter the peroxisomal β-oxidation cycle. Mammalian and yeast peroxisomes contain Lon protease [75,76]. The enzyme has so-called AAA (ATPases associated with diverse cellular activities) domain [77] and apparently involved in cleavage unfolded and non-assembled peroxisomal matrix proteins [76]. Therefore, metabolism in mammalian and yeast peroxisomes requires a continuous flow of ATP into the particles. The molecular size of ATP (507.2 Da) and the strong negative charge of the molecule predict that the transfer of this 'bulky' compound across the peroxisomal membrane is highly restricted (see Section 2 for details). To facilitate this process, the membranes from yeast (Ant1p [28]), mammalian (PMP34 or SLC25A17 [29]), and plant (PNC1 and PNC2 [30,31]) peroxisomes contain solute transporter(s) specific for ATP. These integral membrane proteins belong to a superfamily of mitochondrial solute carriers and accelerate transfer of cytoplasmic ATP into the peroxisomal lumen in exchange for ADP and AMP, which provides a shift in the steady-state concentrations of the nucleotides inside the particles in favor of ATP [28,78].

Solute transporters other than the ATP/AMP antiporter have been suggested to localize in the membrane of mammalian peroxisomes. They include monocarboxylate transporters from rat liver [79], phosphate [80] and 2-ketoglutarate/isocitrate [81] transport systems from bovine kidney. Although the transport activities were detected in isolated peroxisomal fractions, the proteins responsible for these activities were not described. It is noteworthy that the peroxisomal localization of transporters specific for small solutes is difficult to reconcile with the presence of channels open to these solutes (see Section 5). For a discussion on the apparent peroxisomal carnitine transport system and bile acid transporter see Section 8.

7. Peroxisomal shuttle systems

Some membranes closed to solutes such as the inner mitochondrial and chloroplast membranes require shuttle systems to permit the transfer of electrons (reducing equivalents) without physical exchange of the redox cofactors NAD/H and NADP/H between the lumen of organelles and the cytoplasm [82]. As a rule, the shuttle systems include NAD/NADP-dependent dehydrogenases located on both sides of the membrane and solute transporters specific for the electron carriers. Since the membrane of peroxisomes is impermeable to 'bulky' cofactor molecules one can predict that these organelles also require systems that shuttle redox equivalents similar to those found in mitochondria and chloroplasts. Indeed, several such systems have been proposed for yeast [22–24,83], mammalian [1,3,4], and plant [84,85] peroxisomes. However, it seems that in contrast to the shuttle systems of other organelles, the peroxisomal systems exploit channel proteins instead of solute transporters for the transmembrane transfer of electron carriers.

NADH is readily produced in peroxisomes by the bifunctional proteins during β-oxidation of fatty acids and bile acid intermediates [86]. Several other enzymatic activities generating NADH, e.g., xanthine oxidoreductase [87], alcohol [88] and aldehyde [89,90] dehydrogenases, have been described in mammalian peroxisomes. These particles also contain enzymes consuming NADPH including several that catalyze some auxiliary reactions of β-oxidation, i.e. 2,4-dienoyl-CoA reductase for oxidation of (poly)unsaturated fatty acids [86]. In addition, NADPH is required for conversion of 2-phytenoyl-CoA to phytanoyl-CoA — the main substrate for peroxisomal α-oxidation [91], production of ether glycerophospholipids [92,93] and for intraperoxisomal synthesis of the NO free radical [94]. Finally, NADPH is used as a reducing agent to prevent the inactivation of catalase by H_2O_2 [95].

Reoxidation of NADH in mammalian peroxisomes occurs via lactate/pyruvate and glycerophosphate shuttles. The reducing equivalents are transferred from NADH on pyruvate and dihydroxyacetonephosphate (DHAP) by the action of lactate dehydrogenase or NAD-dependent glycerol-3-phosphate dehydrogenase, respectively. A small amount of both enzymatic activities have been detected in rodent liver peroxisomes [96,97]. The resulting products – lactate and glycerol-3-phosphate – are small solutes which easily escape peroxisomes through membrane channels. Then, cytosolic lactate dehydrogenase and FAD-dependent glycerol-3-phosphate dehydrogenase on the outer surface of inner mitochondrial membrane re-oxidize these compounds. In this way the lactate/pyruvate and glycerophosphate shuttles provide a direct flow of electrons from the peroxisomal lumen to the cytosolic compartment and the mitochondrial respiratory chain, respectively. In contrast to mammalian peroxisomes, the re-oxidation of NADH in yeast [22] and plant [84] peroxisomes relies mainly on a malate/oxaloacetate shuttle system.

Reduction of $NADP^+$ in mammalian peroxisomes is ensured by NADP-dependent isocitrate dehydrogenase [98] and glucose-6-phosphate dehydrogenase [99]. Isocitrate dehydrogenase is responsible for the $NADP^+$ reduction in yeast [23,24,83] peroxisomes. Again, as in the case of NAD-dependent dehydrogenases (see earlier discussion), a small portion of these enzymes is localized to peroxisomes whereas the bulk of the activity is detected in the cytosol. However, contrary to the NAD-dependent dehydrogenases, the NADP-dependent enzymes catalyze reactions irreversible under physiological conditions. Hence, the substrates and the products of these

reactions are unable to conduct cycling conversion, i.e., to shuttle. Instead, these reactions in peroxisomes should be considered as shunt systems with respect to the main flow of the corresponding metabolites in the cytosol.

The carnitine shuttle system involved in the transportation of acyl(acetyl)-moieties is discussed in the next section.

8. Export of the products of peroxisomal β-oxidation

An increasing body of evidence indicates that the transmembrane transport of the substrates for peroxisomal β-oxidation (straight very long- and long-chain fatty acids, branched-chain fatty acids, precursors of bile acids and others) is catalyzed by specific half-ABC-transporters [100]. These transport systems are discussed in a separate section of this issue. Here we focus on mechanisms leading to the release of the β-oxidation products from peroxisomes. These mechanisms are quite diverse, and peroxisomes can be considered to be a 'sorting hub' providing cells with the half-finished products, which can be metabolized further in the other cellular compartments (Fig. 2). Peroxisomal β-oxidation of very long- (VLCFA) and long-chain (LCFA) fatty acids in mammals does not proceed to completion but instead is limited to only a few cycles of oxidation resulting in the formation of medium-chain fatty acyl-CoA's and acetyl-CoA (propionyl-CoA in case of the β-oxidation of branched-chain fatty acids) [86]. The other products of peroxisomal β-oxidation in mammals include bile acids and compounds released during degradation of dicarboxylic acids such as malonyl-CoA, succinyl-CoA, and medium chain dicarboxylic acyl-CoA esters. Remarkably, all these compounds are CoA derivatives which, due to their size, are unable to cross the membrane by peroxisomal channels. Therefore, CoA behaves as a keeper of the β-oxidation products inside peroxisomes. Thus, an overall strategy of the peroxisomal transport machinery is to separate the corresponding moieties from CoA and enable them to escape from the particles. To achieve this, peroxisomes exploit several enzymatic systems generating a wide variety of intermediate metabolites.

8.1. Carnitine system

Carnitine (β-hydroxy-γ-trimethylaminobutyrate) is a small and highly hydrophilic molecule that plays a key role in the transfer of acetyl(acyl) moieties between different compartments in eukaryotic cells (reviewed in [101]). The presence of carnitine acetyltransferase that is active towards acetyl-CoA and propionyl-CoA [102] and carnitine octanoyltransferase that is specific for medium-chain acyl-CoA's (the hexanoyl derivative is most preferred) [103] in mammalian peroxisomes indicates the ability of the particles to synthesize acetyl(propionyl)- and acylcarnitines. The size of these compounds are smaller than the corresponding CoA derivatives and they apparently represent shuttle molecules facilitating the release of acetyl(propionyl) and medium-chain acyl groups from peroxisomes. After diffusion across the peroxisomal membrane, apparently via channels, the carnitine esters are then converted back to free carnitine and acetyl(acyl)-CoA's by cytoplasmic and mitochondrial carnitine acyltransferases. Yeast peroxisomes also contain carnitine acyl(acetyl)-transferase that is shown to be a component of the shuttle system that connects the peroxisomal lumen with the cytosolic and the mitochondrial compartments [104–106]. Interestingly, a significant portion of carnitine acetyltransferase is associated with the membrane of rat liver peroxisomes (Antonenkov, unpublished result). Preliminary data indicate that the enzyme is localized on the outer (cytoplasmic) surface of the membrane, which implies that it may be active in the synthesis of acetyl-CoA from

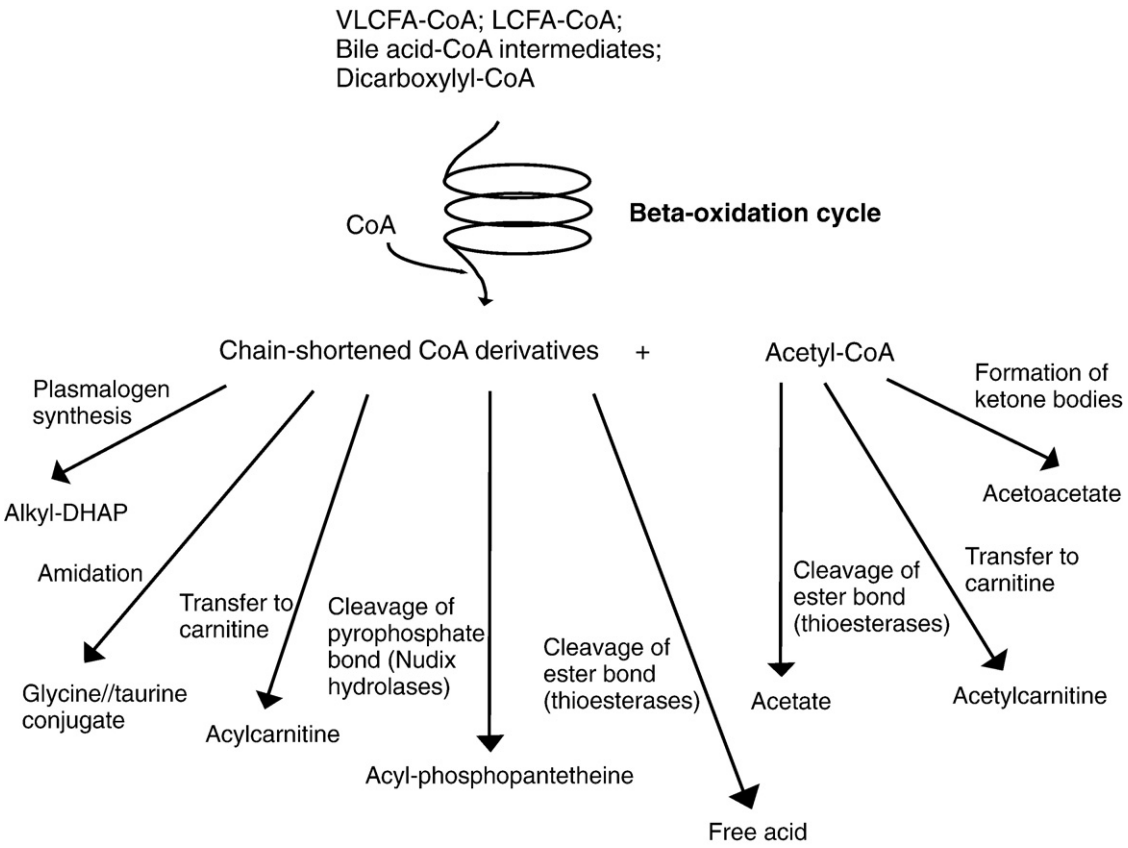

Fig. 2. Release of β-oxidation products from peroxisomes.Peroxisomes harbor a variety of enzymes that catalyze the formation of molecules that are much smaller and mainly more hydrophilic than the initial substrates such as the 'bulky' products of the β-oxidation (chain-shortened CoA esters of fatty and bile acids and acetyl-CoA). Most of these smaller molecules can be released from peroxisomes through membrane channels. See text for more details.

acetylcarnitine and create a driving force to facilitate the transfer of acetylcarnitine out of peroxisomes.

It has been suggested that the cation/carnitine transporter, OCNT3, is present in mammalian peroxisomes [107]. OCNT transporters form a small family of proteins. Three mouse transporters with different affinities for carnitine have been shown to belong to this family [108]. All of them increase the unidirectional transport of carnitine into transfected cells, which suggests the transporters are localized in the plasma membrane. Mouse OCNT3 is highly expressed in the testis and to a much lower extent in kidney, but is not expressed in other tissues including liver [108]. Some information casts doubt on the peroxisomal localization of OCNT3. For instance, the human gene for OCNT3 has not yet been identified. The expression of mouse OCNT3 and its rat homolog, the CT1 protein, are clearly tissue-specific and show preference for the testis [108]. An intriguing question that is as yet unanswered is — how does the peroxisomal protein promote the import of carnitine across the plasma membrane into the whole cell? Finally, attempts to use a genetic approach to detect a peroxisomal carnitine transporter in the yeast *S. cerevisiae* were without success although the plasma membrane carnitine transporter (Agp2p) was readily identified [105].

8.2. Thioesterases

Acyl(acetyl)-CoA thioesterases, known as Acot proteins, comprise a large group of enzymes that catalyze the hydrolysis of acyl(acetyl)-CoA to free fatty acids (or acetic acid) and CoA. The thioesterases are localized in almost all cellular compartments, including the endoplasmic reticulum, cytosol, mitochondria, and peroxisomes. Several excellent reviews have described the structure and function of these enzymes [109–111]. Mouse peroxisomes have been shown to contain six thioesterases: Acot3–6, Acot8, and Acot12. These enzymes differ from each other in substrate specificity. Thus, the preferred substrate for Acot3 is palmitoyl-CoA (C16). Acot4 is active towards succinyl-CoA, one of the products of the β-oxidation of dicarboxylic fatty acids. Acot5 is specific towards medium-chain acyl-CoA esters such as decanoyl-CoA (C10). Acot6 catalyzes the hydrolysis of branched-chain phytanoyl-CoA and pristanoyl-CoA. Acot8 shows very broad substrate specificity and may be involved in the hydrolysis of medium-chain dicarboxylic acyl-CoA esters and CoA derivatives of bile acids. Acot12 is responsible for the hydrolysis of short-chain acyl-CoA's including acetyl-CoA and propionyl-CoA [110]. The role of thioesterases in the modulation of the intraperoxisomal pools of CoA and acyl(acetyl)-CoA esters has been proposed [110,112]. Indeed, at least two (Acot8 and Acot12) out of six peroxisomal thioesterases are regulated by free CoA. On the other hand, it is obvious that the thioesterases create conditions favorable for the transfer of at least some of the β-oxidation products out of peroxisomes. Indeed, hydrolysis by these enzymes of CoA esters results in the formation of small solutes such as acetate, propionate, and succinate, which are conducted effectively by peroxisomal membrane channels. Remarkably, an analysis of the expression rate of short- and medium-chain carnitine acyltransferases and acyl-CoA thioesterases in different mouse tissues revealed that these enzymes provide complementary systems for the transport of β-oxidation products out of peroxisomes [113]. An involvement in the transport function may also be attributed to the yeast *S. cerevisiae* thioesterase Pte1p. The enzyme is essential for the efficient degradation of short-chain and branched-chain fatty acids [114].

8.3. Ketone bodies formation

Ketone bodies (acetoacetate and β-hydroxybutyrate) are synthesized from acetyl-CoA by a short metabolic pathway that involves four consecutive reactions catalyzed by acetoacetyl-CoA thiolase, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase, HMG-CoA lyase, and β-hydroxybutyrate dehydrogenase, respectively. Acetoacetyl-CoA is the first intermediate in the synthesis of ketone bodies. Production of this compound has been registered in isolated peroxisomes from rat liver [115,116]. A wide range of different thiolases, including 3-oxoacyl-CoA thiolases, which are abundantly present in peroxisomes, are able to catalyze the synthesis of acetoacetyl-CoA *in vitro*. However, it is generally believed that only the subclass of thiolases specific for acetoacetyl-CoA (acetoacetyl-CoA thiolases) catalyze the reversible formation of acetoacetyl-CoA from two molecules of acetyl-CoA *in vivo*. Localization of acetoacetyl-CoA thiolase in mammalian peroxisomes has been confirmed independently by two groups [117,118] although the molecular nature of the enzyme has not been established conclusively.

HMG-CoA synthase catalyzes the formation of HMG-CoA from acetoacetyl-CoA. Two genes coding for this enzyme have been identified in mammals [119]. One of the isoforms is targeted to mitochondria whereas the other enzyme is apparently present in both cytosolic and peroxisomal compartments [118].

HMG-CoA lyase catalyzes an irreversible cleavage of HMG-CoA to acetyl-CoA and free acetoacetate, thus producing the ketone body. The peroxisomal localization of this enzyme is well established [120,121]. Mouse and human HMG-CoA lyases contain both the mitochondrial targeting peptide at the N-terminus and the peroxisomal targeting tripeptide (Cys-Lys-Leu) at the C-terminus, respectively. As a result, the same protein is targeted to both organelles [121]. Noteworthy, all ketogenic reactions described earlier use CoA derivatives as substrates. In contrast, the final product of ketogenesis, acetoacetate, is not a CoA ester and therefore is able to leave peroxisomes. Interestingly, our attempts to find the last enzyme of ketogenesis, β-hydroxybutyrate dehydrogenase, in mammalian peroxisomes have failed (Antonenkov, unpublished observation). The enzyme catalyses a reaction in which the substrate (acetoacetate) and the product (β-hydroxybutyrate) do not bound to CoA. This probably eliminates the advantage of the intraperoxisomal localization of β-hydroxybutyrate dehydrogenase.

8.4. Amidation of bile acids and fatty acids

The products of the peroxisomal β-oxidation of C_{27} bile acid precursors (3,7-dihydroxycholestanoic and 3,7,12-trihydroxycholestanoic acids) are the CoA esters of C_{24} bile acids (choloyl-CoA and chenodeoxycholoyl-CoA, respectively) [86,122]. Peroxisomal Acot8 thioesterase is able to convert these esters into free bile acids and CoA [112] apparently facilitating transfer of the bile acids out of peroxisomes. However, one can expect that the main pathway for the export of bile acids from peroxisomes includes their amidation by taurine and glycine catalyzed by bile acid-CoA:amino acid N-acyltransferase (BAAT) [123–125]. The resulting products of the reaction are free CoA and tauro- and glyco-bile acids. The BAAT activity is important to increase the solubility of the bile acids. A significant portion [123,124] if not all [125] the BAAT enzyme is located in liver peroxisomes in mammals. Some recent observations support the conclusion that the BAAT enzyme is not only responsible for the amidation of bile acids produced in peroxisomes but is also involved in the reconjugation of bile acids that have been deconjugated in the intestine and recycled via the enterohepatic circulation [126]. If all the BAAT enzyme is concentrated in peroxisomes and does not show dual, peroxisomal and cytosolic, localization, the flow of the bile acids across the peroxisomal membrane can be expected to be highly intensive. The transmembrane transfer of C_{27} bile acid precursors is apparently catalyzed by the peroxisomal ABC-transporters [100]. However, how C_{24} bile acids including tauro- and glyco-derivatives are transported across the peroxisomal membrane is not yet clear. The role for the peroxisomal channels in the transfer of bile acids is questionable owing to the large size of these molecules. Interestingly, the bile acid transport activity has been

detected using the protein fraction from bovine kidney peroxisomes [127]. It seems that the same transporter accepts both glycocholic and taurocholic acids as substrates. The activity is not dependent on ATP, which indicates that the transporter facilitates passive unidirectional transfer of the bile acids according to their concentration gradient. The protein nature of the peroxisomal bile acid transport activity remains to be established.

In addition to bile acids, the BAAT enzyme is able to catalyze an interaction of fatty acyl-CoA esters with glycine and taurine and the formation of the corresponding fatty acid conjugates and free CoA [128]. But this activity of the BAAT enzyme is low. However, the mouse genome contains two genes (*Acnat1* and *Acnat2*) showing high sequence similarity to the gene (*Baat*) coding the BAAT enzyme. All three genes form a gene cluster on mouse chromosome 4 B3. The human genome contains only two genes in the cluster, one of which is the *BAAT* gene and the other is a pseudogene with predicted stop-codons in the sequence [110]. The protein product of the mouse *Acnat1* gene has recently been characterized as a peroxisomal acyltransferase that effectively conjugates very long- and long-chain fatty acids to taurine with the release of free CoA [129]. Thus, the enzyme may provide a route to transfer long-chain fatty acids out of peroxisomes.

8.5. Formation of plasmalogens

Mammalian DHAPAT is a peroxisomal enzyme involved in the production of ether-linked lipids including plasmalogens as well as triacylglycerols and other non-ether glycerolipids [130–132]. DHAPAT catalyzes the acylation of DHAP by acyl-CoA esters, which can be produced by the β-oxidation of fatty acids. The enzyme is active towards long-chain acyl-CoA esters, e.g., palmitoyl-CoA. The acyl-DHAP esters are converted to the alkyl-DHAP ethers by the peroxisomal enzyme alkyl-dihydroxyacetonephosphate synthase (alkyl-DHAP synthase) which initiates the ether lipid biosynthesis pathway [132–134]. Alkyl-DHAP synthase catalyzes the replacement of the acyl-group of acyl-DHAP with a long-chain fatty alcohol. DHAPAT and alkyl-DHAP synthase form a complex in peroxisomes [135], which apparently facilitates the transfer of the acyl-DHAP between the two enzymes and prevents its escape from the particles before alkyl-DHAP synthesis is complete.

One can expect that the acylation of DHAP is involved in the mechanism responsible for the transmembrane transfer of LCFA from peroxisomes. LCFA may be formed in peroxisomes by the β-oxidation of VLCFA. How acyl(alkyl)-DHAP derivatives cross the peroxisomal membrane is not yet clear. The low solubility of these compounds in water may impedes their transfer by the peroxisomal channels and implies the existence of transporters specific for acyl(alkyl)-DHAP derivatives.

8.6. Products of the β-oxidation of dicarboxylic acids

A substantial part (5–10%) of the total pool of cellular fatty acids is degraded via ω-oxidation. This pathway, which results in the formation of the carboxylic group at the ω-carbon atom of fatty acids, is catalyzed by enzymes of the endoplasmic reticulum [136]. The ω-dicarboxylic acids formed together with dicarboxylic acids derived from food are the substrates for peroxisomal β-oxidation [137,138]. Several products specific for the β-oxidation of dicarboxylic acids are formed in the peroxisomal lumen including medium-chain dicarboxylic acyl-CoA esters, succinyl-CoA, and malonyl-CoA. These last two compounds are the final products of the β-oxidation of even and odd chain-length dicarboxylic acids, respectively. Medium-chain dicarboxylic acyl-CoA's are the substrates for Acot8 thioesterase showing broad substrate specificity whereas succinyl-CoA is hydrolyzed by Acot 4 which is highly specific towards this substrate [112,139]. Malonyl-CoA is converted to acetyl-CoA by peroxisomal malonyl-CoA decarboxylase [140,141]. Acetyl-CoA can be hydrolyzed by Acot12 [112], enter the ketone body formation pathway or the carnitine shuttle (see earlier discussion).

9. Import of cofactors

Cofactors (NAD/H, NADP/H) very slowly diffuse across the rat liver peroxisomal membrane *in vitro* [32] indicating that this is one possible way to deliver these compounds into the particles *in vivo*. The rate of membrane penetration by the cofactors may be enough to avoid exploiting costly transfer mechanisms by transmembrane transporters. The diffusion mechanism predicts the involvement of peroxisomal membrane channels in the transfer of cofactors and, as a result, equilibrium in the concentrations of cofactors inside and outside peroxisomes. The later may decrease the risk of an osmotic imbalance in the particles. However, the extremely slow rate of the transmembrane diffusion of cofactors argues against a direct involvement of this process in the export/import of redox equivalents. These functions rely on the shuttle systems described in peroxisomes (see Section 6). One can expect that these systems ensure a separate functional state of the cofactors inside the particles relative to the surrounding cytosol.

An alternative to a diffusion mechanism is a transmembrane transfer of cofactors by specific transporters. The transporters for CoA molecules in the inner mitochondrial membrane have been described [142,143]. The human solute carrier SLC25A42 catalyzes the import of CoA into mitochondria in exchange for mitochondrial adenine nucleotides, mainly ADP and adenosine 3′,5′-diphosphate [142]. The yeast carrier Leu5p and its human homolog, the Graves' disease protein, also promote the accumulation of CoA in the mitochondrial matrix [143]. The proteins mentioned earlier belong to the superfamily of mitochondrial solute carriers. The primary sequences of the members of this family typically share 25–40% of their amino acid residues, including a characteristic carrier signature motif. At least two members of the family have been detected in peroxisomes: the ATP transporter (see Section 6) and the Ca^{+2}-dependent transporter from rabbit [144]. The sequence of the Ca^{+2}-dependent protein reveals 78% homology to the Graves' disease carrier and 67% homology to the human ADP/ATP translocase. The protein reversibly binds Ca^{+2} with high affinity, which implies a possible regulatory mechanism. Obviously, the high sequence similarity of the peroxisomal Ca^{+2}-dependent carrier to the Graves' disease protein may indicate an involvement of the former in the transmembrane transfer of CoA and/or other cofactors. Interestingly, in a more recent report, the Ca^{+2}-dependent solute carrier from the liver of rats with a 79.1% homology to the rabbit peroxisomal transporter has been described. However, the subcellular localization of this protein was exclusively mitochondrial, not peroxisomal [145]. Deletion of yeast Leu5p leads to a severe reduction in the mitochondrial CoA level. However, the mitochondria still contain low yet significant amounts of CoA implying an alternative although less efficient pathway to carry the cofactor into the matrix [143]. Some authors speculate that the most evident candidates for the role of the secondary CoA transporter are the ADP/ATP carriers [146]. These proteins share the highest sequence similarity with Leu5p within the whole family of yeast solute carriers. Cofactors such as CoA, NAD/H, and NADP/H might be appropriate substrates for ATP transporters owing to the presence in their molecules of an adenine nucleotide residue. An intriguing question that still remains to be answered: does the peroxisomal ATP/AMP antiporter (see Section 6) is able to transfer cofactors side by side with ATP? Interestingly, the peroxisomal ATP transporters show a higher sequence similarity to NAD^+ and FAD carriers than to adenine nucleotide translocases [146].

Quite recently the first peroxisomal transporter specific for coenzymes has been found in plant cells [147]. Apparently, the PXN protein from *Arabidopsis thaliana* facilitates import of cytosolic

NAD^+ into peroxisomes. The loss of PXN in *A. thaliana* causes defects in NAD^+-dependent β-oxidation of fatty acids [147].

One of the options to transfer CoA across the peroxisomal membrane is in the form of acyl-CoA derivatives by means of half-ABC-transporters [100]. According to this mechanism, each transport event delivers one molecule of CoA into peroxisomes. However, as discussed in Section 8, the peroxisomal metabolic machinery functions to release acetyl- and acyl-units from acetyl(acyl)-CoA in the final step of β-oxidation, and to promote diffusion of these units out of the particles leaving free CoA inside the organelles. Therefore, under these conditions peroxisomes can be expected to be overloaded with free CoA.

Peroxisomes might exploit an unusual pathway to import cofactors by carrying out the last reactions in the biosynthesis of these molecules. Two final steps in the synthesis of CoA: (1) the formation of dephospho-CoA from 4′-phosphopantothenic acid and ATP and (2) the phosphorylation by ATP of dephospho-CoA with the production of CoA, are catalyzed by the bifunctional enzyme CoA synthase containing both phosphopantetheine adenylyltransferase and dephospho-CoA kinase domains [148]. Another enzyme, nicotinamide mononucleotide (NMN) adenylyltransferase converts NMN and ATP to NAD^+ [149]. Both enzymes catalyze the formation of large cofactor molecules from the relatively small precursors, phosphopantetheine and NMN, respectively. Therefore, one can expect that these enzymes may serve in peroxisomes to produce 'bulky' cofactors using ATP delivered by a specific transporter whereas phosphopantetheine and NMN may be imported into the particles through membrane channels. Intracellular localization of enzymes catalyzing the formation of cofactors is a matter of controversy. For instance, initial reports indicated localization of rat liver CoA synthase activities in the mitochondrial matrix and in the cytosol [150]. The authors have emphasized that the localization of a portion of CoA synthase in peroxisomes cannot be excluded. However, more recently the enzyme was detected only on the outer mitochondrial membrane and not in the peroxisomes of human cells [151]. Nevertheless, in the proteomic analysis of *Arabidopsis* leaf peroxisomes dephospho-CoA kinase was localized to peroxisomes [152].

According to recent observations, human cells contain three isoforms of NMN adenylyltransferase. One of them is a nuclear protein whereas two others were localized to the Golgi complex and the mitochondria, respectively [153].

10. Elimination of cofactors from peroxisomes – nudix hydrolases

Peroxisomes contain a large set of enzymes that generate reactive oxygen species (ROS) such as H_2O_2, O_2^-, and NO, as by-products of the corresponding reactions [2]. ROS are powerful oxidants and the cofactor molecules are especially vulnerable to the destructive action of these compounds. Therefore, the removal of damaged cofactors from the particles might be important to sustain normal peroxisomal metabolism. Elimination of cofactor molecules from peroxisomes may also contribute to the regulation of their steady-state concentration in the particles. The apparent mechanism responsible for removal of soluble cofactors (NAD/H, NADP/H, and CoA) from the peroxisomal lumen is quite unusual. Most probably it combines the actions of membrane channels and matrix enzymes called Nudix hydrolases. The Nudix family consists of a large group of pyrophosphatases containing a highly conserved amino acid sequence, the Nudix box [154]. The enzymes catalyze the hydrolysis of a pyrophosphate bond connecting nucleosides with a variety of moieties including components of the cofactor molecules. Remarkably, Nudix hydrolases active towards cofactors are localized to peroxisomes. Mammalian NUDT7 [155] and RP2p (NUDT19) [156] enzymes are CoA diphosphatases whereas NUDT12 cleaves NAD(P)/H [157]. Similarly, yeast peroxisomes contain two Nudix hydrolases: Pcd1p [158] and Npy1p [159] catalyzing hydrolysis of CoA and NAD(P)/H, respectively. Peroxisomal Nudix hydrolases cleave pyrophosphate bonds connecting two parts of nearly equal size in 'bulky' cofactor molecules. As a result, the size of the reaction products is approximately half that of the initial substrate, e.g., the reaction catalyzed by NUDT12 or Npy1 is: $NAD^+ + H_2O \rightarrow NMN + AMP$. One can suggest that due to their size the products of cofactor cleavage may freely leave peroxisomes using membrane channels. Peroxisomal Nudix hydrolases show some preference for damaged cofactor molecules indicating their role as 'housecleaning' enzymes. Unexpectedly, the mammalian Nudix hydrolases specific for CoA are active towards some acyl-CoA derivatives. Thus, NUDT19 not only hydrolyzes CoA but cleaves a wide range of CoA-esters including medium chain fatty-acyl-CoA's and choloyl-CoA [160]. Similarly, NUDT7 shows high diphosphatase activity towards bile acid-CoA's and lauroyl-CoA (C_{12}-CoA) [156]. The products of these reactions are acyl(bile acid)-phosphopantetheines and 3,5-ADP. The fate of acyl-phosphopantetheines is not clear. These compounds may comprise one of the ways to release the β-oxidation products from peroxisomes (see Section 8). The other possibility is that they are cleaved by peroxisomal thioesterases with the release of free fatty or bile acids and 4-phosphopantetheine.

11. Conclusion

Information on peroxisomal metabolite transfer that has been collected over the past decade has allowed us to formulate a basic concept regarding the permeability of the peroxisomal membrane to solutes. This concept was instrumental in the discovery of the first peroxisomal membrane channel - Pxmp2. Other important data obtained by different groups of investigators include characterization of the peroxisomal ATP transporter, analysis of substrate specificity of the half-ABC-transporters, and identification of the functions of a large set of enzymes facilitating the export of the β-oxidation products from peroxisomes. However, the aim of the current review is not only to present well-established data but also to discuss ideas that may be helpful in the further study of the transport function of the peroxisomal membrane. From the review it is obvious that future projects will be focused on further characterization of the molecular nature of peroxisomal channels and on the search for proteins responsible for the export from peroxisomes of bile acids, long-chain fatty acids, and plasmalogenes. Other issues that need to be resolved include the transmembrane transfer of oxidized glutathione (see ref. [1] for more detailed discussion) and the role of acyl-CoA synthases in the unidirectional transportation of free fatty acids across the peroxisomal membrane.

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