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Research Article

Solute traffic across mammalian peroxisomal membrane – single-channel conductance monitoring reveals pore-forming activities in peroxisomes

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Abstract. Mouse liver peroxisomes were isolated by centrifugation in a self-generated Percoll gradient followed by an Optiprep density gradient centrifugation. Peroxisomes contributed 90–96% of the total protein content in the fraction, as confirmed by marker enzyme assays, protein pattern in SDS-PAGE, immunoblotting, and electron microscopy. Solubilized peroxisomal membrane proteins were reconstituted into a planar lipid bilayer. A single-channel conductance monitoring of the reconstituted lipid bilayer revealed the presence of two pore-forming components with a conductance in 1 M KCl of 1.3 nS and 2.5 nS. Control experiments with fractions enriched in mitochondria, lysosomes, and fragments of endoplasmic reticulum showed that the peroxisomal channel-forming activities were not due to admixture of isolated peroxisomes with other cellular organelles. The peroxisomal channels were well preserved in membrane preparations but became unstable after solubilization from the membranes by detergent.

Key words. Mammalian peroxisome; membrane; pore-forming channel; single-channel analysis.

Peroxisomes are ubiquitous organelles which contain enzymes and other proteins participating in different metabolic pathways such as α- and β-oxidation of fatty acids, synthesis of bile acids, plasmalogens, and waxes, and oxidation of L-α-hydroxy acids, polyamines, purines, and some amino acids [1–3]. The importance of peroxisomes for cell metabolism is emphasized by the existence of a group of inherited diseases (Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum's disease, rhizomelic chondrodysplasia punctata) caused by impairment of one or more peroxisomal functions [2, 4].

Peroxisomes consist of a matrix containing mostly soluble proteins which is surrounded by a single membrane [1]. The carbon fluxes through peroxisomal pathways require a continuous metabolite crossing of the peroxisomal membrane. A long-standing and still unresolved problem in the physiology of mammalian peroxisomes is the role of the membrane of these organelles as a permeability barrier to solute molecules [reviewed in refs. 3, 5, 6]. A key question is whether metabolites are transferred across the membrane by specific protein translocators, as in the case of inner mitochondrial membrane, or whether

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they penetrate the membrane through large non-selective channels formed by proteins which functionally resemble voltage-dependent anion channels of the outer mitochondrial membrane. Decades of intensive study have resulted in a large set of apparently contradictory results which favor one or other viewpoint.

Based on experiments on isolated peroxisomes we have recently formulated a new conception describing the permeability of the peroxisomal membrane [7]. According to our results, the membrane of rat liver peroxisomes is permeable to small solutes, e. g., urate, glycolate, and other water-soluble peroxisomal metabolites, but it heavily restricts the permeation of more 'bulky' hydrophilic compounds such as cofactors (NAD/H, NADP/H, CoA and its acetyl/acyl derivatives). The hypothesis was able to reconcile a number of apparently conflicting results published thus far [see refs. 7, 8 for more details]. To explain a large difference (over 100-fold [7]) in the rates of penetration across peroxisomal membrane of small metabolites and 'bulky' cofactors, we proposed that the membrane contains at least two types of pore-forming channels. Channels of one type are accessible to small metabolites but completely prevent the penetration of 'bulky' solutes. Channels of the other type are slightly larger and allow the slow diffusion of cofactors and other 'bulky' molecules as well as the high rate of penetration of small metabolites. The relative quantity in the membrane of smaller channels probably exceeds the content of larger ones allowing much faster penetration of small metabolites relative to 'bulky' solutes.

Several reports have described channel-forming activities in peroxisomal preparations isolated from plants [9–11], yeasts [12] and rat liver [13, 14], although the molecular nature of these activities remains obscure. Electrophysiological analysis using fusion of rat liver peroxisomal membrane to planar lipid bilayers [13] or reconstitution of this membrane in liposomes [14] demonstrated the presence of a voltage-dependent, cation-selective channel with an estimated diameter of 1.5–3.0 nm. It was speculated that this channel might account for the permeability of the peroxisomal membrane to solutes including cofactors [15]. However, the presence of pore-forming proteins in mammalian and yeast peroxisomes was later challenged [16–23] (see Discussion for details).

In the present study, we applied an electrophysiological approach to detect pore-forming channels in a highly purified peroxisomal fraction isolated from mouse liver. Reconstitution experiments on lipid bilayer membranes using detergent-solubilized peroxisomal membrane preparations showed the presence of two pore-forming components with a single-channel conductance of 1.3 nS and 2.5 nS in 1 M KCl. Some properties of these channels were examined. The possible role of the peroxisomal membrane in the transfer of different metabolites is discussed.

Materials and methods

Materials. Percoll (colloidal suspension of silica) was purchased from Amersham. Diphytanoyl phosphatidylcholine was from Avanti Polar Lipids. Genapol X-080 and Epoxy-embedding medium were from Fluka. Criterion Precast Gels were from Bio-Rad. Fixatives and other reagents for electron microscopy were from Electron Microscopy Sciences. The sources of antibodies are indicated in the text. All other chemicals were of analytical grade and obtained from Sigma.

Animals and isolation of highly purified peroxisomes and mitochondria. The use of experimental animals was approved by the committee on animal experimentation at the University of Oulu. Male C57BL/6J mice weighting 25–30 g were fasted overnight. Animals were sacrificed with an overdose of carbon dioxide. To obtain highly purified peroxisomes we used a procedure described previously for rat liver [8] with some modifications. The livers (10–12 g) were homogenized in isolation medium 1 (1:5, w/v) containing: 0.16 M sucrose, 12% (w/v) PEG 1500, 10 mM MOPS, pH 7.4, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, 0.1% (v/v) ethanol, and 0.1 mM PMSF. In some experiments, the isolation medium for purification of peroxisomes and mitochondria (see below) contained a cocktail of protease inhibitors (set III) from Calbiochem (one 1-ml vial per 500 ml of isolation medium). The homogenates were centrifuged at 500 g_{max} for 10 min and then at 1200 g_{max} for 12 min. The two-step centrifugation was applied to prevent admixture of purified peroxisomes with erythrocytes from blood. The resulting supernatant was centrifuged at 23,000 g_{max} for 20 min to yield sediment containing mainly mitochondria and peroxisomes. The pellet was resuspended in the isolation medium (total volume 80 ml) and the suspension was loaded on a layer of 50% (w/v) Percoll solution (100 ml). The centrifugation of the Percoll and the subsequent Optiprep gradients was conducted exactly as described before [8]. The Optiprep gradients were fractionated starting from the bottom, yielding 14 fractions per gradient. Fractions enriched in peroxisomes (see 'Results') were combined and diluted fourfold with the isolation medium. The organelles were then sedimented by centrifugation at 100,000 g_{max} for 45 min. The organelle pellets were stored at –80 °C until use. In some experiments, the peroxisomal 'ghosts' were prepared by sonication of purified peroxisomes in 50 mM Tris-Cl, pH 8.2 containing 0.15 M KCl. The 'ghosts' were separated from matrix proteins by centrifugation at 100,000 g_{max} for 60 min or by using multistep sucrose gradient centrifugation [24]. Sonication (three cycles, 15 s each; amplitude 15 µm) was performed as described previously [8].

To isolate purified mitochondria, the livers (6–8 g) were homogenized in isolation medium 2 (1:5, w/v) contain-

ing 0.25 M sucrose, 10 mM MOPS, pH 7.4, 1 mM EDTA, 2 mM DTT, and protease inhibitor cocktail. The homogenates were centrifuged at 1000 g_{max} for 5 min and the mitochondria were sedimented at 15,000 g_{max} for 10 min [25]. The sediment was resuspended in 80 ml isolation medium 2 and loaded onto a layer of 50% (w/v) Percoll solution (100 ml) prepared on the same medium. The Percoll gradient was generated by centrifugation at 23,500 g_{max} for 60 min at slow acceleration and deceleration. Fractions enriched in mitochondria were collected from the bottom of the gradient (total volume 16 ml) and diluted with 8 ml of isolation medium 2. An aliquot (8–9 ml) of this suspension was layered on the top of an Optiprep gradient prepared as described before [8]. The gradients were centrifuged at 65,000 g_{max} for 1 h in a vertical rotor VTi50 (Beckman) using slow acceleration and deceleration modes. The described procedure exploits the high fragility of peroxisomes in solutions containing sucrose only as an osmoprotector [see ref. 8 for more details]. Under these conditions, osmotically broken peroxisomes form membrane 'ghosts', poor in matrix proteins. The 'ghosts' are characterized by a lower equilibrium density relative to intact peroxisomes and collect mainly on the top of a Percoll gradient [8]. The organelles which are sedimented near the bottom of the Percoll gradient (mainly mitochondria, lysosomes, and intact peroxisomes) can be separated further by Optiprep gradient centrifugation.

Marker enzyme assays. Activities of marker enzymes for the subcellular organelles: mitochondria (glutamate dehydrogenase), lysosomes (acid phosphatase), endoplasmic reticulum (esterase), and peroxisomes (L-α-hydroxyacid oxidase) were measured according to standard procedures [24, 26, 27].

Planar lipid bilayer assay. The methods used for channel-forming activity detection are described in detail elsewhere [28]. The measurements were conducted using a Teflon cell separated by a thin wall containing a circular hole with a surface area of 0.2 mm^2. Both compartments were filled with 5 ml of unbuffered 1 M KCl. The bilayer membranes covering the hole were formed from a 1% (w/v) solution of diphytanoyl phosphatidylcholine in n-decane. Peroxisomal membranes were solubilized in 0.5% (w/v) Genapol X-080. An insoluble material was sedimented by centrifugation at 100,000 g_{max} for 45 min and the resulting preparation containing solubilized membrane proteins was immediately used for detection of the channel-forming activity. Peroxisomal suspension (5 µl, 0.5–1.5 µg protein/ml) was added to both sections of the chamber. The voltage dependence of the channels was assessed using membrane potentials as high as –100 to + 100 V.

Electron microscopy. For transmission electron microscopy, isolated organelles were fixed in 1% (w/v) glutaraldehyde as described before [8]. After fixation, the particles were sedimented by centrifugation. The pellets were postfixed with 1% (w/v) osmium tetroxide and stained with 1% (w/v) uranyl acetate. The samples were then dehydrated and embedded in Epoxy-embedding medium. Thin sections were made throughout the pellet, contrasted with uranyl acetate and lead citrate, and examined in a Philips EM410 transmission electron microscope.

Other methods. The composition of subcellular fractions was examined by SDS/PAGE using 15% (w/v) Criterion Precast Gels. Protein bands were visualized by silver staining. Immunoblotting was performed using a semi-dry blotter and the blots were incubated with the primary antibodies followed by detection with alkaline-phosphatase-labeled anti-rabbit or anti-goat IgG [29]. Polyclonal antibodies used were against catalase and glutamate dehydrogenase from bovine liver (Chemicon), the recombinant N-terminal segment of human NADPH: cytochrome P450 reductase (Santa Cruz Biotech), a synthetic peptide corresponding to amino acid residues 185–197 of human voltage-dependent anion channel, isoform-1 (VDAC1) (Oncogene), and against a peptide mapping near the carboxy terminus of human VDAC2 (Santa Cruz Biotech). Antibodies against synthetic peptides corresponding to the N terminus of mouse peroxisomal membrane protein with a monomeric molecular mass of 22 kDa (PMP22) (NH_2-APAASRLRVESELG) and the C terminus of rat liver inner mitochondrial membrane marker D-3-hydroxybutyrate dehydrogenase (THFPGAISDKIYIH-COOH) were prepared by standard procedures [30]. Protein was determined according to Bradford [31].

Results

Characterization of the peroxisomal fraction. Highly purified peroxisomes were isolated from mouse liver using medium containing PEG 1500 and density gradient centrifugation [8]. Peroxisomes constituted 90–96% (n = 3) of the total protein content in the combined Optiprep gradient fractions 3–6 (fig. 1) as was estimated by marker enzyme activity determination as described elsewhere [27]. The data were confirmed by electron microscopic examination of the purified peroxisomal fraction (fig. 2A, B). The images obtained from different sections of the organellar pellet were analyzed (see Materials and methods). The fraction almost exclusively consisted of peroxisomes. Some of them were intact (structures with high electron density, fig. 2A, B) while others represented peroxisomal 'ghosts' with a poor content of matrix proteins. The latter indicates partial damage of peroxisomes during isolation. On several micrographs, a low mitochondrial

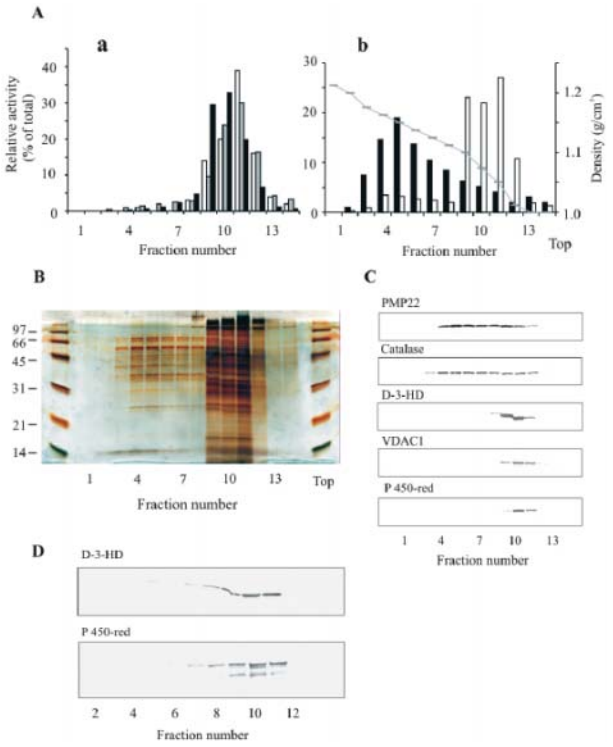

Figure 1. Separation of peroxisomes from other cellular organelles by Percoll and Optiprep gradient centrifugation. (*A*) Fractions from the Optiprep gradient were analyzed for the following marker enzyme activities: glutamate dehydrogenase (a, filled bars), acid phosphatase (a, gray bars), esterase (a, open bars), L-α-hydroxyacid oxidase (b, filled bars), or protein content (b, open bars). Results are expressed as relative activity (relative protein content) in each fraction (% of the total activity loaded on the gradient). Enzyme (protein) recoveries varied between 84–106%. (*B*, *C*) Proteins from equal volumes of each fraction were separated by SDS-PAGE and silver-stained (*B*) or immunoblotted (*C*). Molecular-mass markers are indicated on the *left* in *B*. Note (*A, C*) clear separation of peroxisomes (fractions 3–6, markers: L-α-hydroxyacid oxidase, PMP22, and catalase) from mitochondria [markers: glutamate dehydrogenase, D-3-hydroxybutyrate dehydrogenase (D-3-HD), and VDAC1], microsomes [markers: esterase and NADPH:cytochrome P450 reductase (P450-red)], and lysosomes (marker: acid phosphatase). In contrast, fractions 9–11 enriched in mitochondria and microsomes were contaminated by peroxisomes. Immunodetection of VDAC2 showed the same pattern in the gradient fractions as for VDAC1 (data not shown). (*D*) Immunoblots of D-3-HD and P 450-red were overexposed to detect traces of mitochondria and microsomes in the gradient fractions containing mainly peroxisomes (fractions 3–7). The difference between fractions in the migration pattern of D-3-HD (see *C*, *D*) is explained by the shift in the rate of protein migration through the corresponding region of the gel (see *B*). This shift is probably due to a large difference in the total protein content in fractions 3–8 and 9–12 (see *B*).

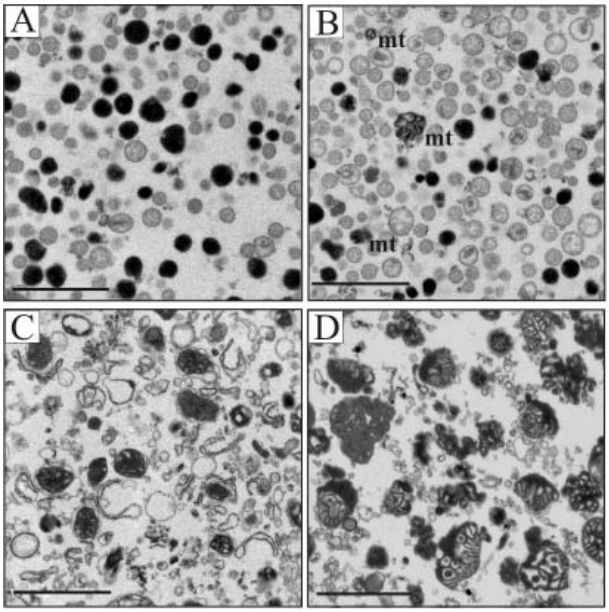

Figure 2. Electron micrographs of subcellular organelles obtained after Optiprep gradient centrifugation. Gradient fractions enriched in peroxisomes (fractions 3–6, see fig. 1) or mitochondria and microsomes (fractions 9–11) were combined and mixed with an equal volume of 2% (w/v) glutaraldehyde prepared on 40% (w/v) Optiprep solution to avoid osmotic damage of peroxisomes [see ref. 8 for further details]. The same procedure was applied to the purified mitochondrial fraction. After overnight fixation, the organelles were sedimented and processed for electron microscopy. Typical images are presented. Original magnification was x 3800, bar 1000 nm. (*A, B*) The bottom gradient fractions (see fig. 1) contained highly purified peroxisomes, many of them heavily filled with electron-dense matrix; mitochondria (mt) are barely detectable. (*C*) The fractions from the top of the gradient were enriched in mitochondria and fragments of endoplasmic reticulum, and also contain structures resembling peroxisomal 'ghosts'. (*D*) Purified mitochondrial fraction.

contamination was detected (marked on fig. 2B). The result indicated that peroxisomes were well separated from other cellular organelles including mitochondria which may represent a potential source of contaminating pore-forming activities by VDAC isoforms. The distribution of organelles in the final Optiprep gradients was monitored by measuring the marker enzyme activity in the fractions (fig. 1A), by observing the protein pattern in the silver stained SDS-PAGE gels (fig. 1B), or by immunoblotting using antibodies generated against peroxisomal membrane (PMP22) and matrix (catalase) proteins as well as markers for mitochondrial matrix (glutamate dehydrogenase) (data not shown), outer (VDAC1) and inner [3-D-hydroxybutyrate dehydrogenase (D-3-HD)] membranes and for endoplasmic reticulum [NADPH:cytochrome P450 reductase (P450-red)] (fig. 1C). According to enzymatic analysis, contamination of gradient fractions enriched with peroxisomes (fractions 3–6 on fig. 1A) by mitochondria and microsomes was very limited, and corresponded to the results of electron microscopic examination. The visualized protein pattern of these fractions was similar to that described previously [8, 32, 33] for highly purified peroxisomal preparations isolated from rodent liver (fig. 1B). We were unable to detect immunoblot signals of mitochondrial and microsomal marker

proteins in the gradient fractions containing mainly peroxisomes at standard conditions of Western blotting (fig. 1C, fractions 4–7). However, when immunoblots of D-3-HD and P 450-red were overexposed, some traces of these marker proteins were found in the peroxisomal fractions (fig. 1D). In contrast to purified peroxisomes, the top of the Optiprep gradient (fractions 9–11, see fig. 1) consisted of a mixture of different organelles including mitochondria and fragments of endoplasmic reticulum as well as lysosomes and structures resembling peroxisomal 'ghosts' (fig. 2C).

To discriminate further peroxisomal and mitochondrial pore-forming activities, we prepared a purified mitochondrial fraction with a low admixture of peroxisomes (see Materials and methods). As shown in figure 2D, this fraction consisted mainly of mitochondria with some admixture of lysosomes; peroxisomes were barely detectable. The enrichment of glutamate dehydrogenase was 4.8-fold while specific activity of peroxisomal L-α-hydroxyacid oxidase was reduced 2.8-fold in the purified mitochondrial fraction relative to homogenate.

Detection of the pore-forming activity. By using single-channel analysis, we investigated if purified peroxisomes contained any pore-forming components and found that the peroxisomal preparations isolated from mouse liver displayed two major types of a channel-forming activity with an average conductance of 1.3 nS and 2.5 nS in 1 M KCl (fig. 3A, B, panel 1). To study further the peroxisomal nature of the observed main channels, we compared pore-forming activities of the peroxisomal fraction with the reported activities in mitochondria. Therefore, testing was carried out with the pooled Optiprep gradient fractions 9–11 (see fig. 1) enriched in mitochondria. The most frequent pore-forming events were with a conductance of 4.0 nS in 1 M KCl (fig. 3B, panel 2). This conductance is known to be characteristic for the mitochondrial porin in its open state [34]. Of note, in the same experiment, the frequency of the pore-forming events with a conductance of 1.3 nS and 2.5 nS was much lower than in the peroxisomal fraction. The observed channel-forming activity with a conductance of 2.0 nS in 1 M KCl may be caused by the mitochondrial porin in its closed configuration [34]. However, we were unable to detect a correlation between the relative proportions of mitochondria or peroxisomes and the frequency of the 2.0 nS insertion events. Because the pore-forming activity with a conductance of 2.0 nS was detected also in peroxisomal preparations (see fig. 3B, panels 1 and 3), the peroxisomal membrane may house a third channel-forming activity with a conductance very similar to that of the mitochondrial porin in the closed state. The fifth channel-forming activity registered in the pooled 9–11 gradient fractions showed a low average conductance (0.5 nS in 1 M KCl). It was not detected in the peroxisomal fraction (see fig. 3B, panel 2; compare to panel 1). The nature of this channel is not clear.

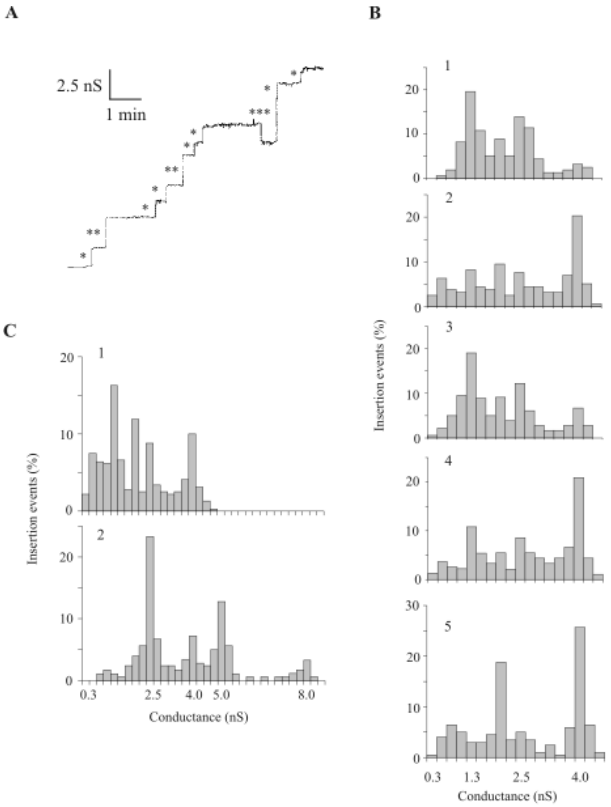

Figure 3. Detection of the channel-forming activity. (*A*) Single-channel recording of artificial membrane in the presence of detergent-solubilized peroxisomes purified from mouse liver. Insertion events with an average conductance 1.3 nS (*), 2.5 nS (**), and 4.0 nS (***) in 1 M KCl are marked. The addition of detergent alone at a similar concentration to that used with the protein had no effect on the membrane conductance. The aqueous phase contained 1 M KCl. The temperature was 20 °C, and the applied voltage was 20 mV. (*B*) Histograms of the individual single-channel conductance events observed in the presence of freshly prepared detergent-solubilized peroxisomes (panel 1; combined gradient fractions 3–6, see fig. 1); fraction enriched in mitochondria and microsomes (panel 2; combined gradient fractions 9–11, see fig. 1); peroxisomal 'ghosts' (panel 3); purified peroxisomes which were solubilized by 0.5% (w/v) Genapol X-080 and then kept overnight at +4 °C (panel 4); purified mitochondrial fraction (panel 5). (*C*) Detection of the pore-forming activity of the sample consisting of aliquots of the gradient fractions 3–8 (see fig. 1) at 1 M KCl (panel 1) and 2 M KCl (panel 2) in an aqueous phase. The total number of insertion events was 150–170 for each membrane preparation analyzed. Each experiment was repeated three to five times; typical pictures are presented.

To confirm the peroxisomal origin of the pore-forming activities with conductance 1.3 nS and 2.5 nS in 1 M KCl, we tested a purified mitochondrial fraction (fig. 3B, panel 5). As expected for this fraction, the bulk of pore-forming events represents activity of VDAC in its open (4.0 nS in 1 M KCl) and closed (2.0 nS in 1 M KCl) states. Pore-forming events with a conductance

of 1.3 nS and 2.5 nS in 1 M KCl were also detected, but their frequency was much lower relative to the activity of mitochondrial porin. To prevent apparent proteolysis of peroxisomes and mitochondria during their isolation and storage, some pore-forming activity measurements were performed on fractions purified in the presence of a protease inhibitor cocktail (see Materials and methods). The results were the same as those obtained under standard conditions (data not shown).

Further experiments were conducted to reveal the localization of the channel-forming activities in peroxisomes. The particles were disrupted by sonication followed by centrifugation in a multistep sucrose gradient yielding peroxisomal 'ghosts' enriched in membrane but depleted of matrix proteins [see ref. 8 for details]. After treatment of the 'ghosts' by detergent and sedimentation of an insoluble material by high-speed centrifugation, the supernatant containing solubilized membrane proteins was analyzed for channel-forming activity. Similar to the findings with the peroxisomal preparations, the activities with a conductance of 1.3 nS and 2.5 nS in 1 M KCl were detected (fig. 3, panel 3). In separate experiments, we were unable to register any pore-forming activity in the fractions from sucrose gradients containing peroxisomal matrix proteins. As a whole, the data indicate that the pore-forming activities are localized in the peroxisomal membrane.

Properties of the peroxisomal channels. The measurements on freshly prepared peroxisomal preparations solubilized with Genapol X-080 showed step changes in the membrane conductance mainly with an average level of 1.3 nS and 2.5 nS in 1 M KCl (see fig. 3A). The vast majority of the steps were directed toward an increase in the conductance, indicating that the peroxisomal channels were stable in the lipid bilayer. The pore-forming activities were well preserved by keeping purified peroxisomes or peroxisomal 'ghosts' at –70 °C. However, the activities became unstable after membrane solubilization by detergents. Overnight storage at +4 °C of the peroxisomal preparations solubilized by Genapol X-080 or Triton X-100 was accompanied by a large decline in the frequency of the insertion events with conductances of 1.3 nS and 2.5 nS. In the same preparations, the pattern of peroxisomal proteins detected by silver staining was not visibly affected during storage, indicating that factors other than proteolysis may influence activity of the solubilized channels. The decrease in peroxisomal pore-forming activities resulted in the detection of more frequent pore-forming events corresponding to channels from contaminating organelles (fig. 3, panel 4; compare to panel 1).

The simultaneous presence of two channel-forming activities in purified peroxisomal preparations complicated their separate electrophysiological analysis. Preliminary measurements conducted on the peroxisomal fraction showed that the channel-forming activities exhibited no voltage dependence even at voltages as high as ±100 V, indicating the occurrence of separate pore-forming proteins, rather than different states of the same channel (voltage-dependent gating) [28, 34]. Analysis of the ion selectivity showed that the overall pore-forming activity in the peroxisomal fraction is cation selective, with a P_K/P_{Cl} ratio, estimated in KCl gradients, close to 4 (data not shown). Registration of the single-channel conductance of peroxisomal preparations in the presence of 3.0 M KCl in an aqueous phase revealed the two main types of channels with an average conductance of 3.8 nS and 7.5 nS. The result indicates that both peroxisomal channels show linear dependence of a single-channel conductance on KCl concentration.

Studies on the channel-forming activities of peroxisomal preparations at different KCl concentrations revealed an unusual feature of the two peroxisomal channels: the amount of their insertion into the lipid bilayer increased with an increase in KCl concentration in an aqueous phase. Under the assay conditions used, we were unable to register any channel-forming activity (except a very low mitochondrial porin admixture) in the freshly isolated peroxisomal preparation at 0.3 M KCl. In the same preparation of peroxisomes, the rate of insertion events with a conductance of 1.3 nS and 2.5 nS in 1 M KCl was relatively low: less than ten insertion events per 40 min for each type of channel. A further increase in KCl concentration up to 3.0 M led to the appearance of an intensive channel-forming activity with a single-channel conductance of 3.8 nS and 7.5 nS. In this case, however, quantification of pore-forming events was difficult owing to frequent disruption of the membrane bilayer. An approximate estimation showed that the frequency of the insertion events increased three- to fourfold over the experiments conducted at 1.0 M KCl. The influence of KCl concentration on the rate of pore-forming activity has been observed before for colicin E1 from *Escherichia coli* [35] and for the channel from spinach leaf peroxisomes [11].

To analyze the effect of KCl concentration on the relative frequency of peroxisomal pore-forming events, fractions 3–8 of the Optiprep gradient (see fig. 1) were combined and the resulting preparation containing in addition to peroxisomes some admixture of mitochondria and other organelles was used for single-channel monitoring in the presence of 1 M KCl (fig. 3C, panel 1) or 2 M KCl (fig. 3C, panel 2). The pattern of insertion events at 1 M KCl reflected the presence of both peroxisomal and mitochondrial pore-forming activities in the preparation at roughly similar proportions. However, the pattern of insertion events at 2 M KCl showed a clear predominance of peroxisomal channel-forming activities with an average conductance of 2.5 nS and 5.0 nS over the activities

with conductance 4.0 nS and 7.5–8.0 nS, which could be attributed to mitochondrial porin (VDAC from mammalian mitochondria shows a linear dependence of conductivity on KCl concentration [36]). The data indicate that the peroxisomal pore-forming activity may be 'latent' at low KCl concentrations, but is easily detectable at higher salt concentrations.

Discussion

Our results demonstrate that the mammalian peroxisomal membrane contains at least two channel-forming activities with different properties from those of other cellular channels, e. g., VDAC of the outer mitochondrial membrane. The channel-forming activities showed a single channel conductance of 1.3 nS and 2.5 nS (1 M KCl). This conductance differs from that formed by mitochondrial porins in open (4.0 nS) or closed (2.0 nS) configurations under otherwise identical conditions [34]. Noteworthy is that in contrast to the mitochondrial VDAC, the peroxisomal channels were found not to be voltage gated. Mitochondrial VDAC forms a large diffusion pore with an estimated diameter of 2.5–3.0 nm that allows unimpeded diffusion through the channel of not only small metabolites but also more 'bulky' solutes such as cofactors (NAD/H, NADP/H and CoA) and ATP [34]. The data of single-channel measurements suggest that the pore diameter of both peroxisomal channels is smaller than that of the mitochondrial VDAC. This may indicate that the peroxisomal channels heavily restrict the rate of penetration of bulky solutes into the particles or are even impermeable to these compounds.

Attempts to detect a channel-forming activity in mammalian (rat liver) peroxisomal membranes have been made previously [13, 14]. The authors described a large voltage-gated channel with a conductance in an open state of 2.4 nS in 0.3 M KCl [13] or with a conductance of 0.4 nS in 0.1 M KCl [14]. The estimated size of these channels (1.5–3.0 nm) is comparable to that of mitochondrial porins [34]. Since purified peroxisomal fractions are always more or less contaminated by mitochondria, it is reasonable to expect the presence of a VDAC-dependent pore-forming activity in the peroxisomal preparations tested. This activity may be misinterpreted as truly peroxisomal. In the studies cited, the peroxisomal preparations were incubated in alkaline solutions (pH 9.0 or 11.5) to obtain a fraction enriched in integral membrane proteins. According to our data [unpublished results], such treatments cause partial inactivation of the pore-forming activities registered in mouse liver peroxisomes.

The main factor negatively influencing the detection of peroxisomal channels is the extremely high lability of these channels after their solubilization by detergent. Another feature of the peroxisomal channels that might hamper determination of the pore-forming activity is the dependence of this activity on the KCl concentration in an aqueous phase.

The pore-forming activity with a single-channel conductance of 0.35 nS in 1 M KCl has been described in the membrane of leaf peroxisomes [9, 11]. Despite the difference in size, the mammalian and leaf peroxisomal channels possess some similar properties: they are voltage independent and highly unstable after solubilization from the membranes.

Several indirect observations favor the presence of pore-forming channels in mammalian peroxisomes: (i) the absence of the structure-linked latency of some peroxisomal oxidases (urate oxidase, oxidase D-amino acids, L-α-hydroxyacid oxidase) [7, 26, 37]; (ii) rapid, temperature-independent incorporation of radioactive compounds (carnitine, sucrose, NAD^+, ATP, etc.) into isolated peroxisomes [15]; (iii) leakage of radioactive sucrose from liposomes reconstituted with peroxisomal membrane proteins [15]. However, some experimental results have been interpreted as non-consistent with the presence of porin-like channels in peroxisomes from mammals and yeasts. The most frequent objections refer to the apparent existence of a pH gradient across the peroxisomal membrane. In situ experiments depending on the approaches used have shown that the lumen of yeast peroxisomes is acid [16, 38] or basic [39]. The alkaline pH was registered in mammalian peroxisomes [20] while the results of another study [40] demonstrated that the peroxisomal membrane is freely permeable to protons (H^+). The controversy surrounding the intraperoxisomal pH may indicate that the data described above depend mostly on the experimental approaches exploited rather than reflecting the real conditions in the peroxisomal lumen. Moreover, it is not necessary to consider the impermeability of the peroxisomal membrane to small solutes as an obligatory condition for the formation of a transmembrane pH gradient. The difference in the pH levels in the peroxisomal lumen and in surrounding cytoplasm may be created by the mechanism known as a Donnan equilibrium [41, 42]. This phenomenon is responsible, while only partially, for the maintenance of an acid pH in lysosomes [43] and for some other cellular parameters [44, 45]. The presence of charged macromolecules (proteins) in peroxisomes and the low permeability of mammalian peroxisomal membrane to 'bulky' solutes such as cofactors and ATP, which are mainly charged compounds, may be responsible for the formation of a pH gradient between the peroxisomal lumen and the cytoplasm. This suggestion predicts that the intraperoxisomal pH may be a dynamic parameter which heavily depends on the physiological conditions in the cell.

The presence of at least two types of channel in the mammalian peroxisomal membrane has recently been

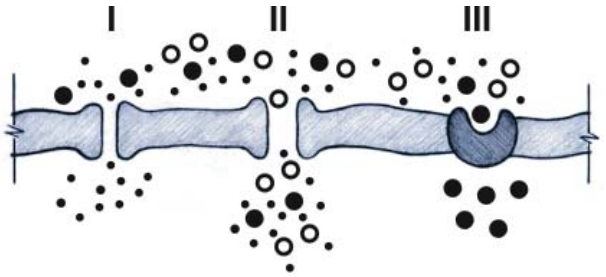

Figure 4. Two-channel model of peroxisomal membrane permeability to solutes. To explain a difference of over 100-fold [see ref. 7], in the rate of peroxisomal membrane permeation to small metabolites (small ●) and 'bulky' molecules such as cofactors (○) or ATP (large ●) we proposed the presence in the membrane of at least two types of pore-forming channel. The smaller channel allows rapid diffusion of small metabolites through the membrane barrier but is impermeable to 'bulky' molecules (I). It is more abundant in the membrane than the larger channel (II), which allows the slow diffusion of 'bulky' molecules as well as the rapid penetration of small metabolites. The model is compatible with the presence of active transporters in the peroxisomal membrane specific for 'bulky' molecules, e. g., an ATP/AMP antiporter (III), which has been characterized recently [21, 22]. The rapid 'pumping' of 'bulky' compounds by active transporters into peroxisomes (III) may exceed the rate of slow diffusion of these solutes through the larger channel (II) out of the particles. This will create a higher steady-state concentration of 'bulky' compounds inside versus outside the organelle.

predicted by us to explain unusual permeability properties of this membrane in vitro which are clearly different from those of other eukaryotic membranes (fig. 4) [see also ref. 7]. The rat liver peroxisomal membrane is freely permeable to solutes with a molecular mass less than 300 Da while strongly impeding the penetration of compounds comparable in size to cofactors (600–800 Da). Most probably, this is the way peroxisomes are able to keep in vivo their own, functionally independent pool of cofactors and, at the same time, share a common pool of small metabolites with the surrounding cytoplasm.

Our 'two-channel' hypothesis provides an easy explanation for the presence of specific metabolite translocators in the peroxisomal membrane (see fig. 4). One of these translocators has recently been described as an ATP-dependent ATP/AMP antiporter [21, 22]. The presence of metabolite transporters side by side with non-specific pore-forming channels in the same peroxisomal membrane may be advantageous only if the transporters carry 'bulky' solutes with a very low rate of free transmembrane diffusion through the channels, e. g., cofactors and ATP. Under these conditions, the active transport of some 'bulky' solutes into peroxisomes may exceed the level of their free diffusion out of the particles and provide a shift to a higher steady-state concentration of these compounds inside peroxisomes relative to surrounding cytoplasm.

Four proteins related to the superfamily of ABC transporters have been described in the mammalian peroxisomal membrane [reviewed in refs. 46, 47]. These proteins, called ALDP, ALDR, PMP 70, and PMP 69, interact *in situ* with each other forming heterodimers. Mutations in the gene coding for ALDP cause a severe neurodegenerative X-linked disorder adrenoleukodystrophy [4]. The substrate specificity and molecular mechanism of the peroxisomal ABC transporters are not clear. However, a large set of indirect observations points out the role of these proteins in the translocation across the peroxisomal membrane of lipid compounds poorly soluble in water, e. g., very long chain fatty acids [reviewed in refs. 3, 5, 6, 46, 47]. Therefore, the ABC transporters most probably fulfill functions which are not carried out by the peroxisomal membrane channels.

Three transporters specific to small solute molecules have been suggested to localize in the mammalian peroxisomal membranes [23, 48, 49]. Two of them, carnitine-acylcarnitine carrier [48] and monocarboxylate transporter [49], were detected in the peroxisomal preparations isolated from rat liver using antibodies generated against corresponding mitochondrial proteins. However, the purity of the peroxisomal fractions used in these experiments was poorly characterized, especially in terms of mitochondrial admixture. No further analysis of the transporters has been reported. More recently, phosphate transporter activity has been detected in peroxisomes isolated from bovine kidney [23]. Proteoliposomes containing peroxisomal membrane proteins were able to take up radioactive phosphate while the reaction did not show saturation at elevated concentrations of the substrate (saturation behavior is an intrinsic property of transmembrane translocators). In addition, the peroxisomal activity was not suppressed by known inhibitors of phosphate transporters. These observations imply that phosphate may penetrate the peroxisomal membrane by a mechanism other than specific translocation. Moreover, active phosphate uptake by proteoliposomes reconstituted with peroxisomal membrane proteins is difficult to reconcile with previously published results obtained using a similar experimental approach [15]. The results showed that proteoliposomes loaded with radioactive sucrose are leaky if they contain proteins from rat liver peroxisomal membrane.

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1 Masters C. and Crane D. (1995) The Peroxisomes: A Vital Organelle. Cambrige University Press, Cambridge
2 Mannaerts G. P., Van Veldhoven P. P. and Casteels M. (2000) Peroxisomal lipid degradation via beta- and alpha-oxidation in mammals. Cell Biochem. Biophys. **32:** 73–87
3 Hiltunen J. K., Mursula A. M., Rottensteiner H., Wierenga R. K., Kastaniotis A. J. and Gurvitz A. (2003) The biochemistry of

peroxisomal beta-oxidation in the yeast Saccharomyces cerevisiae. FEMS Microbiol. Rev. **27:** 35–64
4 Wanders R. J., Schutgens R. B. and Barth P. G. (1995) Peroxisomal disorders: a review. J. Neuropathol. Exp. Neurol. **54:** 726–739
5 Wanders R. J., Vreken P., Ferdinandusse S., Jansen G. A., Waterham H. R., Roermund C. W. T. van et al. (2001) Peroxisomal fatty acid alpha- and beta-oxidation in humans: enzymology, peroxisomal metabolite transporters and peroxisomal diseases. Biochem. Soc. Trans. **29:** 250–267
6 Roermund C. W. van, Waterham H. R., Ijlst L. and Wanders R. J. (2003) Fatty acid metabolism in *Saccharomyces cerevisiae*. Cell. Mol. Life Sci. **60:** 1838–1851
7 Antonenkov V. D., Sormunen R. T. and Hiltunen J. K. (2004) The rat liver peroxisomal membrane forms a permeability barrier for cofactors but not for small metabolites in vitro. J. Cell Sci. **117:** 5633–5642
8 Antonenkov V. D., Sormunen R. T. and Hiltunen J. K. (2004) The behavior of peroxisomes in vitro: mammalian peroxisomes are osmotically sensitive particles. Am. J. Physiol. Cell Physiol. **287:** C1623–C1635
9 Reumann S., Maier E., Benz R. and Heldt H. W. (1995) The membrane of leaf peroxisomes contains a porin-like channel. J. Biol. Chem. **270:** 17559–17565
10 Reumann S., Bettermann M., Benz R. and Heldt H. W. (1997) Evidence for the presence of a porin in the membrane of glyoxysomes of castor bean. Plant Physiol. **115:** 891–899
11 Reumann S., Maier E., Heldt H. W. and Benz R. (1998) Permeability properties of the porin of spinach leaf peroxisomes. Eur. J. Biochem. **251:** 359–366
12 Sulter G. J., Verheyden K., Mannaerts G., Harder W. and Veenhuis M. (1993) The in vitro permeability of yeast peroxisomal membranes is caused by a 31 kDa integral membrane protein. Yeast **9:** 733–742
13 Labarca P., Wolff D., Soto U., Necochea C. and Leighton F. (1986) Large cation-selective pores from rat liver peroxisomal membranes incorporated to planar lipid bilayers. J. Membr. Biol. **94:** 285–291
14 Lemmens M., Verheyden K., Van Veldhoven P., Vereecke J., Mannaerts G. P. and Carmeliet E. (1989) Single-channel analysis of a large conductance channel in peroxisomes from rat liver. Biochim. Biophys. Acta **984:** 351–359
15 Van Veldhoven P. P., Just W. W. and Mannaerts G. P. (1987) Permeability of the peroxisomal membrane to cofactors of beta-oxidation: evidence for the presence of a pore-forming protein. J. Biol. Chem. **262:** 4310–4318
16 Nicolay K., Veenhuis M., Douma A. C. and Harder W. (1987) A 31P NMR study of the internal pH of yeast peroxisomes. Arch. Microbiol. **147:** 37–41
17 Roermund C. W. van, Elgersma Y., Singh N., Wanders R. J. and Tabak H. F. (1995) The membrane of peroxisomes in Saccharomyces cerevisiae is impermeable to NAD(H) and acetyl-CoA under in vivo conditions. EMBO J. **14:** 3480–3486
18 Roermund C. W. van, Hettema E. H., Kal A. J., Berg M. van den, Tabak H. F. and Wanders R. J. (1998) Peroxisomal beta-oxidation of polyunsaturated fatty acids in *Saccharomyces cerevisiae*: isocitrate dehydrogenase provides NADPH for reduction of double bonds at even positions. EMBO J. **17:** 677–687
19 Roermund C. W. van, Hettema E. H., Berg M. van den, Tabak H. F. and Wanders R. J. (1999) Molecular characterization of carnitine-dependent transport of acetyl-CoA from peroxisomes to mitochondria in *Saccharomyces cerevisiae* and identification of a plasma membrane carnitine transporter, Agp2p. EMBO J. **18:** 5843–5852
20 Dansen T. B., Wirtz K. W., Wanders R. J. and Pap E. H. (2000) Peroxisomes in human fibroblasts have a basic pH. Nat. Cell Biol. **2:** 51–53
21 Palmieri L., Rottensteiner H., Girzalsky W., Scarcia P., Palmieri F. and Erdmann R. (2001) Identification and functional reconstitution of the yeast peroxisomal adenine nucleotide transporter. EMBO J. **20:** 5049–5059
22 Visser W. F., Roermund C. W. van, Waterham H. R. and Wanders R. J. (2002) Identification of human PMP34 as a peroxisomal ATP transporter. Biochem. Biophys. Res. Commun. **299:** 494–497
23 Visser W. F., Roermund C. W. van, Ijlst L., Hellingwerf K. J., Wanders R. J. and Waterham H. R. (2005) Demonstration and characterization of phosphate transport in mammalian peroxisomes. Biochem. J. **389:** 717–722
24 Antonenkov V. D. (1989) Dehydrogenases of the pentose phosphate pathway in rat liver peroxisomes. Eur. J. Biochem. **183:** 75–82
25 Graham J. M. (1999) Purification of a crude mitochondrial fraction by density-gradient centrifugation. In: Current Protocols in Cell Biology, Suppl. 4, pp. 3.4.1–3.4.3, Wiley, New York
26 Leighton F., Poole B., Beaufay H., Baudhuin P., Coffey J. W., Fowler S. et al. (1968) The large-scale separation of peroxisomes, mitochondria, and lysosomes from the livers of rats injected with triton WR-1339: improved isolation procedures, automated analysis, biochemical and morphological properties of fractions. J. Cell Biol. **37:** 482–513
27 Fujiki Y., Hubbard A. L., Fowler S. and Lazarow P. B. (1982) Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. J. Cell Biol. **93:** 97–102
28 Ludwig O., De Pinto V., Palmieri F. and Benz R. (1986) Pore formation by the mitochondrial porin of rat brain in lipid bilayer membranes. Biochim. Biophys. Acta **860:** 268–276
29 Novikov D. K., Vanhove G. F., Carchon H., Asselberghs S., Eyssen H. J., Van Veldhoven P. P. et al. (1994) Peroxisomal beta-oxidation: purification of four novel 3-hydroxyacyl-CoA dehydrogenases from rat liver peroxisomes. J. Biol. Chem. **269:** 27125–27135
30 Sambrook J., Fritsch E. F. and Maniatis T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn, pp. 18.3–18.10., Cold Spring Harbor Laboratory Press, Cold Spring Harbor
31 Stoscheck C. M. (1990) Quantitation of protein. Methods Enzymol. **182:** 50–68
32 Alexson S. E., Fujiki Y., Shio H. and Lazarow P. B. (1985) Partial disassembly of peroxisomes. J. Cell Biol. **101:** 294–304
33 Bodnar A. G. and Rachubinski R. A. (1991) Characterization of the integral membrane polypeptides of rat liver peroxisomes isolated from untreated and clofibrate-treated rats. Biochem. Cell Biol. **69:** 499–508
34 Benz R. (1994) Permeation of hydrophilic solutes through mitochondrial outer membranes: review on mitochondrial porins. Biochim. Biophys. Acta **1197:** 167–96
35 Bullock J.O., Kolen, E.R. and Shear, J.L. (1992) Ion selectivity of colicin E1. II. Permeability to organic cations. J. Membrane Biol. **128:** 1–16
36 De Pinto V., Ludwig O., Krause J., Benz R. and Palmieri F. (1987) Porin pores of mitochondrial outer membranes from high and low eukaryotic cells: biochemical and biophysical characterization. Biochim. Biophys. Acta **894:** 109–119
37 Baudhuin P. (1969) Liver peroxisomes, cytology and function. Ann. N. Y. Acad. Sci. **168:** 214–228
38 Lasorsa F. M., Scarcia P., Erdmann R., Palmieri F., Rottensteiner H. and Palmieri L. (2004) The yeast peroxisomal adenine nucleotide transporter: characterization of two transport modes and involvement in DeltapH formation across peroxisomal membranes. Biochem. J. **381:** 581–585
39 Roermund C. W. van, Jong M. de, Ijlst L., Marle J. van, Dansen T. B., Wanders R. J. et al. (2004) The peroxisomal lumen in *Saccharomyces cerevisiae* is alkaline. J. Cell Sci. **117:** 4231–4237
40 Jankowski A., Kim J. H., Collins R. F., Daneman R., Walton P. and Grinstein S. (2001) In situ measurements of the pH of mam-

malian peroxisomes using the fluorescent protein pHluorin. J. Biol. Chem. **276:** 48748–48753
41 Alberts B., Johnson A., Lewis J., Raff M., Roberts K. and Walter P. (2002) Molecular Biology of the Cell, 4nd edn, pp. 615–657, Garland Science, New York
42 Price N. C., Dwek R. A., Ratcliffe R. G. and Wormald M. R. (2001) Principles and Problems in Physical Chemistry for Biochemists, 3nd edn, pp. 132–134, Oxford University Press, Oxford
43 Moriyama Y., Maeda M. and Futai M. (1992) Involvement of a non-proton pump factor (possibly Donnan-type equilibrium) in maintenance of an acidic pH in lysosomes. FEBS Lett. **302:** 18–20
44 Morrill G. A., Kostellow A. B., Osterlow K. and Gupta R. K. (1996) Differences in hydration state of nucleus and cytoplasm of the amphibian oocyte. J. Membr. Biol. **153:** 45–51
45 Wong P. (2002) A hypothesis of haemolysis in haemolytic anaemias associated with enzymopathies. Med. Hypoth. **59:** 105–109
46 Hettema E. H. and Tabak H. F. (2000) Transport of fatty acids and metabolites across the peroxisomal membrane. Biochim. Biophys. Acta **1486:** 18–27
47 Borst P. and Elferink R. O. (2002) Mammalian ABC transporters in health and disease. Annu. Rev. Biochem. **71:** 537–592
48 Fraser F., Corstorphine C. G., Price N. T. and Zammit V. A. (1999) Evidence that carnitine palmitoyltransferase I (CPT I) is expressed in microsomes and peroxisomes of rat liver: distinct immunoreactivity of the N-terminal domain of the microsomal protein. FEBS Lett. **446:** 69–74
49 McClelland G. B., Khanna S., Gonzalez G. F., Butz C. E. and Brooks G. A. (2003) Peroxisomal membrane monocarboxylate transporters: evidence for a redox shuttle system? Biochem. Biophys. Res. Commun. **304:** 130–135