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Visser, W.F.

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Investigations into the permeability of the peroxisomal membrane by measurements of the osmotic behaviour of proteoliposomes containing reconstituted peroxisomal membrane protein

Wouter F. Visser†, Carlo W.T. van Roermund†, Lodewijk Ijlst†, Klaas J. Hellingwerf§, Hans R. Waterham†, Ronald J.A. Wanders†

† Laboratory of Genetic Metabolic Diseases, Department of Clinical Chemistry and Pediatrics, Emma Children’s Hospital, Academic Medical Centre, University of Amsterdam, PO Box 22700, 1100 DE Amsterdam, The Netherlands
§ Swammerdam Institute for Life Sciences, BioCentrum Amsterdam, Faculty of Science, University of Amsterdam, Amsterdam, The Netherlands.

Introduction

Although much has been learned about the metabolic functions of the peroxisome [1-3], much less is known about the transport of metabolites across the peroxisomal membrane. It was initially believed that the peroxisomal membrane is freely permeable to small-molecular weight compounds. This was based on the following observations. Firstly, it was reported that none of the peroxisomal enzymes known at the time exhibited structure-linked latency [4]. Although some latency was observed for catalase, this was explained as being due to its high concentration in the peroxisomal matrix, in combination with its high rate of catalysis. Secondly, it was found that a range of radiolabelled small-molecular weight compounds have free access to the intraperoxisomal space [5]. It was proposed that the peroxisomal membrane contains a large pore-forming protein, or ‘porin’ [6]. Subsequent studies did indeed provide some evidence supporting the existence of a peroxisomal channel [7-12], but this presumed ‘porin’ has not been identified to date.

In contrast, it has been found more recently that the peroxisomal membrane must be impermeable to some substrates, at least in vivo. The phenotype of specific Saccharomyces cerevisiae gene disruption mutants indicates that the peroxisomal membrane is impermeable to NADP(H), NAD(H) and acetyl-CoA. Most likely, the transport of reducing equivalents across the peroxisomal membrane is accomplished by shuttle systems [13-15], while acetyl-units are exported in the form of a carnitine ester or by entering the glyoxylate cycle [14, 16], at least in S. cerevisiae. The transporters involved in the translocation of these metabolites have remained unknown thus far, however. Furthermore, the transport of adenine nucleotides is accomplished by a member of the mitochondrial carrier family of solute transporters (MCF / SLC25), which is located in the peroxisomal membrane. The yeast and human orthologues of this protein (called Ant1p and PMP34 respectively) have recently been characterized by reconstitution in proteoliposomes [17, 18].

It has been speculated that the restricted permeability of the peroxisomal membrane in vivo inferred from the phenotype of S. cerevisiae mutants is lost upon isolation of the organelles to explain the apparent conflict with the previous finding that the peroxisomal membrane appears permeable due to the presence of a large channel. This view led to a number of reports in which peroxisomal latency was measured under conditions that may preserve the integrity of the peroxisomal membrane [19-22]. Antonenko et al recently reported structure-linked latency for some of the peroxisomal matrix enzymes, all of which require a cofactor of relatively large molecular-weight (e.g. NADP(H), NAD(H), CoA) [22]. They proposed that the peroxisomal membrane is permeable to low-molecular
weight compounds, but virtually impermeable to compounds of large dimensions (such as NADP(H), NAD(H), CoA derivatives and adenine nucleotides), thus providing a possible explanation for the requirement of specific transporters even if the membrane is highly permeable to small molecular weight metabolites.

Recently, reconstitution of peroxisomal membrane proteins has yielded evidence to suggest the existence of proteins that facilitate the passage of phosphate [23] and 2-ketoglutarate across the peroxisomal membrane. In both cases, it was observed that transport could proceed unidirectionally, although it was slightly stimulated by the presence of a countersubstrate. Unidirectional transport, through a channel protein or otherwise, may be detected by observations of the osmotic behaviour of proteoliposomes containing reconstituted channel proteins (or intact organelles) upon suspension in iso-osmotic solutions. This method has proven useful for the detection and identification of pore-forming proteins in bacterial membranes [24-29], for the characterization of aquaporins and aquaglyceroporins [30, 31], and for the characterization of transport across the mitochondrial membrane [32]. Therefore, we anticipated that this method could be applied to yield insight into the permeability of the peroxisomal membrane.

Our data obtained using the method described in this chapter suggest that reconstitution of peroxisomal membrane protein into proteoliposomes confers permeability to a range of metabolites. However, these proteoliposomes were found to be impermeable to several large molecular weight compounds including NADPH, NADH, AMP, and oxidized glutathione (GSSG). In light of previous results obtained using radiolabeled compounds to detect the presence of transporters for phosphate and 2-ketoglutarate, the results are best explained by the existence of multiple transport proteins, that cooperate to facilitate the passage of a wide range of compounds.

**Experimental**

**Materials**

Egg-yolk phospholipids were obtained from Fluka (Buchs, Switzerland). Nycodenz was purchased from Nycomed (Oslo, Norway). Biobeads SM-2 was obtained from Biorad (Hercules CA, USA). Sephadex G-75 was from Amersham biosciences (Freiburg, Germany). PMP70 antibody was purchased from Zymogen. All other chemicals were of analytical grade and obtained from Sigma-Aldrich (St. Louis MO, USA)

**Purification of peroxisomes**

A fresh bovine kidney was obtained from a local abattoir and immediately immersed in ice-cold SEME buffer (250 mM sucrose, 1 mM EDTA, 50 mM MOPS pH=7.4, 0.1% v/v ethanol). The cortex was manually separated from other tissue, and homogenised with a Potter-Elvehjem homogenizer (5 strokes at 500 rpm). A postnuclear supernatant was obtained by centrifugation for 5 minutes at 2400* g. From this postnuclear supernatant, a crude organellar fraction was obtained by centrifugation for 30 minutes at 15,000* g. This pellet was subjected to Nycodenz gradient centrifugation [33]. The gradients were harvested from the bottom, yielding 24 fractions per gradient. All fractions were diluted four-fold with SEME buffer, and the organelles were then pelleted by centrifugation (30 minutes, 20,000* g) for storage. The supernatants obtained after centrifugation were found not to contain activity of any of the marker enzymes tested (glutamate dehydrogenase, cytochrome c oxidase, catalase, esterase and alkaline phosphatase) and were subsequently discarded. The organelle pellets were stored at −80°C until use. Prior to reconstitution into proteoliposomes, a peroxisomal membrane fraction was prepared by sonicating an aliquot of the purified peroxisomal fraction in a buffer containing 1M sodium chloride and 25 mM sodium phosphate pH=7.4, followed by high
speed centrifugation (60 minutes at 100,000×g). Sonication was performed on ice using a probe tip sonicator with a power output of 9 watts (Sonic & Materials Inc., Danbury CT., USA; Model VC60), for 4 periods of 15 seconds each, interrupted by intervals of 45 seconds.

**Preparation of proteoliposomes**

Peroxisomal membrane proteins were reconstituted by removal of detergent using an hydrophobic column as described earlier [34], with the following modifications. 50 µg of peroxisomal membrane protein was mixed with 112 µl 10% (w/v) Egg-Yolk phospholipids in the form of sonicated liposomes, 100 µl of 10% (w/v) Triton X-114, 50 mM stachyose, 5 mM HEPES/KOH pH=7.2 and water to a final volume of 700 µl. This mixture was passed 16 times through an Amberlite column (5.0x0.5cm) pre-equilibrated with a buffer containing 50 mM stachyose and 5 mM HEPES pH=7.2. All steps were performed at 4°C, except the passages through the Amberlite column, which were done at room temperature.

**Proteoliposome swelling assay**

The swelling assay was conducted as described by Nikaido *et al* [26, 27], with the following modifications. 50 µl of proteoliposome suspension was rapidly diluted into 950 µl of an isotonic solution of the solute to be tested. The optical density of the suspension was subsequently monitored at a wavelength of 400 nm for a period of 1 minute. It was found that stirring was not required, since the liposomes did not precipitate noticeably within the timescale of the measurements. The isosmotic concentration of a solute was determined by suspending control liposomes, which did not contain protein, into solutions of increasing concentrations of the solute to be tested until a solution was found that did not produce any change in optical density with time, indicating that the liposomes did not exhibit swelling or shrinking. This solution was considered iso-osmotic with the internal solution of the liposomes (50 mM stachyose, 5 mM Hepes pH 7.2). To correct for slight variations in liposome concentration and refractive index of the solutions used, the optical density was normalized with respect to its value at the start of the experiment.

**Results**

*Stachyose does not permeate proteoliposomes containing peroxisomal membrane proteins*

The tetrasaccharide stachyose is frequently used as the internal substrate in liposome-swelling assays since it does not permeate the majority of known channels due to its large dimensions (molecular
weight 667). To verify that stachyose could be used for the same purpose in proteoliposomes containing peroxisomal proteins, proteoliposomes loaded with 50 mM stachyose were rapidly diluted into solutions of dextran T10, and the ensuing change in optical density was monitored for a period of 1 minute. A shown in figure 1, control liposomes, which did not contain any protein, and proteoliposomes containing peroxisomal membrane proteins exhibit identical osmotic behaviour. Since neither stachyose nor dextran T10 is normally able to diffuse across a lipid bilayer at any significant rate, this indicates that the membrane of the proteoliposome is also impermeable to these compounds. Since initial experiments had indicated that dextran T10 interferes with the preparation of proteoliposomes by means of an Amberlite column (not shown), stachyose was used as the internal substrate for all subsequent experiments.

Figure 2: (A) Change in optical density of proteoliposomes after rapid suspension into isotonic solutions of stachyose, sodium 2-ketoglutarate and sodium isocitrate. The proteoliposomes were prepared using 50 μg peroxisomal protein. The optical density is normalized with respect to its initial value to correct for slight differences in liposome concentration. The experiment was conducted four times, the data shown are from a representative experiment. (B) Change in optical density of proteoliposomes containing 20 μg or 40 μg of peroxisomal protein, 40 μg of heat-treated protein and control liposomes (without protein) after rapid dilution into iso-osmotic sodium 2-ketoglutarate solution. The experiment was conducted three times, the data shown are from a representative experiment. (C) Change in optical density of proteoliposomes and control liposomes containing 2-ketoglutarate in the internal volume upon dilution into iso-osmotic stachyose solution.
Figure 3: Time-dependent change in optical density of a proteoliposome suspension after rapid dilution of proteoliposomes containing 40 μg peroxisomal protein in iso-osmotic solutions of stachyose, sodium 2-ketoglutarate and the sodium salts of NADPH and NADH. The optical density was normalized with respect to its initial value to correct for slight differences in liposome concentration. The experiment was conducted three times, the data shown is a representative experiment.

Proteoliposomes containing peroxisomal membrane proteins are permeable to 2-ketoglutarate and isocitrate
Proteoliposomes containing peroxisomal membrane proteins were prepared, containing 50 mM stachyose in the internal compartment. In parallel, control liposomes were prepared which did not contain protein but which were loaded with the same internal buffer. Using control liposomes, the iso-osmotic concentration of various compounds was determined by varying the concentration of the solute until a solution was found that did not result in shrinking nor swelling of the liposomes. As mentioned, the phenotype of certain S. cerevisiae mutants strongly suggests that the peroxisomal membrane contains a transport system that facilitates the passage of isocitrate and 2-ketoglutarate (chapter 5). Therefore, to test the permeability of the membrane of proteoliposomes containing peroxisomal membrane protein to 2-ketoglutarate and isocitrate, these proteoliposomes were rapidly diluted into iso-osmotic solutions of sodium 2-ketoglutarate and sodium isocitrate. The optical density was subsequently monitored for a period of 1 minute. As shown in figure 2A, a rapid decrease in optical density was observed with both isocitrate and 2-ketoglutarate, indicating that significant swelling occurs in these solutions. The protein-dependency of the observed swelling was examined in more detail using sodium 2-ketoglutarate. The rate of swelling as well as the final level were found to be approximately proportional to the amount of protein used for reconstitution (figure 2B). Heat treatment of the peroxisomal protein sample prior to reconstitution (5 minutes at 95°C) was found to disrupt the effect (figure 2C). To test the possibility that the observed decrease in optical density is due to an enzymatic reaction resulting in the formation of a product that results in increased absorption at 400 nm, proteoliposomes were prepared containing sodium 2-ketoglutarate as the internal substrate, and diluted into iso-osmotic stachyose. This reversal of the internal and external substrates resulted in a protein-dependent increase in optical density, indicating shrinkage of the proteoliposomes rather than swelling (figure 2D). This confirms that the change in optical density is due to the movement of 2-ketoglutarate across the proteoliposomal membrane.

Proteoliposomes containing peroxisomal membrane proteins are not permeable to NADH and NADPH
As discussed, the phenotype of certain S. cerevisiae mutants strongly suggests that the peroxisomal membrane is impermeable to NAD(H) and NADP(H). Therefore, using the same procedure, the ability of NADP+ and NADPH to enter proteoliposomes containing peroxisomal membrane protein was tested. Interestingly, dilution of proteoliposomes into iso-osmotic solutions of sodium NADH or sodium NADPH was did not result in any significant change in optical density (figure 3). This
suggests that the proteoliposomal membrane is impermeable to these compounds.

**Substrate specificity**

Based on our current understanding of peroxisomal metabolism, a number of substrates were selected that may be expected to be transported across the peroxisomal membrane in vivo. In all cases, the sodium salt of these compounds was used to prepare an iso-osmotic solution in which control liposomes without protein do not produce any significant time-dependent change in optical density. The rate of swelling of proteoliposomes containing peroxisomal membrane protein in each of these solutions was measured, and the results are summarized in figure 4. As shown, proteoliposome swelling was observed with most of the tested solutes. However, virtually no swelling was observed using the following compounds: NADPH, NADH, oxidised glutathione (GSSG) and AMP. An obvious common characteristic of these compounds is their relatively high molecular weight, ranging from 347 for AMP to 745 for NADPH.

To further test the influence of the molecular weight on the permeability of a compound, the rate of swelling in solutions of polyethyleneglycol (PEG) of increasing molecular weight was measured.
Figure 5 depicts the rate of swelling as a function of the molecular weight of the PEG used. As shown, a decrease in the rate of swelling is observed with increasing molecular weight. The rate of swelling approaches zero beyond approximately 1500 Da, indicating that this represents the approximate exclusion limit, at least for PEG molecules.

Discussion

The results presented in this chapter indicate that the peroxisomal membrane contains one or several proteins to facilitate the passage of a range of compounds. Reconstitution of highly purified peroxisomal membrane proteins in liposomes renders the membrane permeable to many small molecular weight solutes. Interestingly, however, reconstitution of peroxisomal membrane proteins does not confer permeability to NADP(H), NAD(H), AMP or GSSG.

Although it was shown that the mammalian peroxisomal membrane contains a specific transporter for adenine nucleotides, called PMP34, this transport was found to take place exclusively via an exchange reaction. Unidirectional transport of adenine nucleotides is not facilitated by PMP34, thus providing an explanation for the observation that proteoliposome swelling is not observed upon dilution into iso-osmotic AMP solution. Unfortunately, other adenine nucleotides could not be tested using the proteoliposome swelling assay, since the presence of these compounds in the assay medium results in rapid aggregation of the proteoliposomes (not shown). Previous results obtained utilizing radiolabeled substrates suggest that the translocation of (pyro)phosphate [23] and 2-ketoglutarate (chapter 5 of this thesis) can also take place unidirectionally, in agreement with the results obtained using the proteoliposome swelling assay.

A number of substrates were found to produce swelling which were observed not be a substrate for the 2-ketoglutarate transport system by means of a competitive inhibition experiment (chapter 5). Therefore, a plausible explanation for the combined results seems that the peroxisomal membrane contains multiple transport proteins, that cooperate to facilitate the passage of a range of substrates. Combined, these findings provide a possible explanation for the apparent conflict between the long-held view that the peroxisomal membrane is permeable to metabolites, and the presence of shuttle systems and transporters to facilitate the transport of certain metabolites in vivo.
References


