Metabolite transport across the peroxisomal membrane
Visser, W.F.
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Demonstration and characterization of phosphate transport in mammalian peroxisomes

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Introduction

Peroxisomes are involved in a number of important metabolic pathways, including fatty acid α- and β-oxidation, biosynthesis of etherphospholipids and bile acids, and the degradation of purines, amino acids and polyamines [1]. Their importance is underscored by the existence of a number of severe human diseases (e.g. Zellweger syndrome, Rhizomelic chondrodysplasia punctata, X-linked Adrenoleukodystrophy, Refsum’s disease), in which there is a defect in one or more peroxisomal functions [2, 3].

In recent years, much has been learned about the permeability properties of the peroxisomal membrane. Although the membrane of isolated peroxisomes obtained through cell fractionation appears to be freely permeable to small molecules [4-7], several lines of evidence indicate that in vivo the peroxisomal membrane is impermeable to a number of substrates and products of peroxisomal metabolism [8-15]. These findings strongly suggest the existence of specific transport systems. However, only one peroxisomal transporter has currently been characterized to any significant extent, which is the adenine nucleotide transporter Ant1p / PMP34 [16, 17].

In addition to PMP34, the peroxisomal membrane contains several other proteins that bear strong homology to transporters. In mammalian peroxisomes four half ABC transporters have been identified: the adrenoleukodystrophy protein (ALDP), the ALDP-related protein (ALDR), the 70-kDa peroxisomal membrane protein (PMP70), the PMP70-related protein (P70R) [18-21]. However, the exact function of these proteins has remained unresolved thus far.

Presumably, adenine nucleotide transport by PMP34 is required to sustain the activity of the peroxisomal matrix enzyme very-long-chain-acyl-CoA synthetase [22, 23]. However, activity of this enzyme also produces pyrophosphate, which is not normally able to traverse a lipid bilayer at a significant rate due to its polar nature. Therefore, we anticipated that the peroxisomal membrane must also contain a pyrophosphate and/or phosphate transport system that is required to support intraperoxisomal fatty acid activation. In this paper, we demonstrate the presence of a specific (pyro)phosphate transporter activity in the membrane of Bos taurus kidney peroxisomes by reconstitution in proteoliposomes. We have determined several kinetic parameters and provide evidence that this transporter activity is distinct from the mitochondrial phosphate transporter.

Materials and Methods

Materials
Egg-yolk phospholipids were obtained from Fluka (Buchs, Switzerland). Nycodenz was purchased.
Pyrophosphate transport

from Nycomed (Oslo, Norway). Biobeads SM-2 was obtained from Biorad (Hercules CA, USA). Sephadex G-75 and [32P]-phosphate were from Amersham biosciences (Freiburg, Germany). PMP70 antibody was purchased from Zymed (San Fransisco CA, USA). All other chemicals were of analytical grade and obtained from Sigma (St. Louis MO, USA).

Preparation of highly purified peroxisomes

A fresh B. taurus 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 2400xg. From this postnuclear supernatant, a crude organellar fraction was obtained by centrifugation for 30 minutes at 15,000xg. This pellet was subjected to Nycodenz gradient centrifugation [24]. 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, 20,000xg) 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. In some experiments peroxisomal membrane preparations were used, which were 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 100,000xg). Sonication was performed on ice using a probe tip sonicator with a power output of 9 watts (Sonics & Materials Inc., Danbury CT., USA; Model VC60), for 4 periods of 15 seconds each, interrupted by intervals of 45 seconds.

Marker enzyme assays

Protein concentrations were determined with the bicinchoninic acid method [16]. Glutamate dehydrogenase was determined as the 2-ketoglutarate-dependent rate of NADH oxidation (A440 nm) in a mixture containing 50 mM triethanolamine/HCl pH 8.0, 2.5 mM EDTA, 100 mM NH₄Cl, 1 mM ADP, 0.1% v/v Triton X-100, 0.3 mM NADH and 2.5 mM 2-ketoglutarate. Cytochrome c oxidase activity was determined according to the method described by Cooperstein et al [25]. Alkaline phosphatase activity was measured as described by Bowers et al[26]. Esterase activity was determined by monitoring the p-nitrophenol formation (A405 nm) in a mixture containing 50 μM EDTA, 50 mM potassium phosphate buffer pH 6.6, 0.05% Triton X-100, and 2.5 mM p-nitrophenyl acetate. PMP70 was determined by SDS-PAGE followed by Western blotting using a commercial antibody directed against human PMP70, which was found to exhibit cross-reactivity with the B. taurus protein.

Proteoliposome assay

Reconstitution and transport assays were performed as described previously [17] with minor modifications. 250 μg of protein was mixed with 112 μl 10% w/v Egg-Yolk phospholipids in the form of sonicated liposomes, 0.4 mg cardiolipin (sodium salt), 100 μl of 10% w/v Triton X-114, 20 mM sodium chloride, 10 μM sodium fluorescein, 50 mM of potassium phosphate pH=6.5, 20 mM HEPES pH=6.5 and water to a final volume of 700 μl. This mixture was passed 14 times through a Amberlite column (5.0x0.5cm) pre-equilibrated with a buffer containing 20 mM sodium chloride, 10 μM sodium fluorescein, 50 mM potassium phosphate pH=6.5 and 20 mM HEPES pH=6.5. All steps were performed at 4°C, except the passages through the Amberlite column, which were done at room temperature. External substrate was removed from the proteoliposomes on a Sephadex G-75 column equilibrated with a buffer containing 70 mM NaCl and 20 mM HEPES pH=6.5. The reaction was...
Figure 1: Distribution of marker enzymes in the fractions of a *B. taurus* kidney Nycodenz gradient. The marker enzymes measured indicate the distribution of peroxisomes (catalase), mitochondrial matrix (glutamate dehydrogenase), microsomes (esterase), mitochondrial inner membrane (cytochrome c oxidase) and plasma membrane (alkaline phosphatase). The activity is expressed as a percentage of the total activity of all fractions.

started by the addition of 3 mM $[^{33}P]$-labelled phosphate (15 MBq mmol$^{-1}$) to the proteoliposomes, followed by incubation at 30°C for 10 minutes, except where otherwise indicated. The external radioactivity was removed by passing the reaction mixture through a Sephadex G-75 column, thereby stopping the assay, and the radioactivity associated with the proteoliposome fraction was measured by liquid scintillation counting. The stop time was taken as the moment of addition to the sephadex column.

Fluorescein was included in the vesicles to determine the internal volume. For this purpose, a 100 µl sample of the liposome suspension was suspended in a cuvet containing 900 µl 0.1% Triton X-100 to obtain a clear solution and to release the fluorescein from the liposomes. Previous experiments showed that Triton X-100 has no effect on the fluorescence of sodium fluorescein up to a concentration of at least 0.5% (not shown). The fluorescence was measured using an Aminco PA-256-E1 spectrofluorometer (excitation 494 nm / emission 518 nm). A calibration curve ranging from 0 – 0.2 µM fluorescein was included in the experiment in order to allow calculations to be made.

Figure 2: Uptake of radiolabeled phosphate by proteoliposomes containing various amounts of highly purified (total) peroxisomal protein. The data shown are from a representative experiment. Virtually identical results were found in 2 additional experiments.
Results

Purification of B. taurus peroxisomes
Highly purified peroxisomes were obtained through Nycodenz gradient centrifugation of a crude organellar fraction. Marker enzymes were measured in all fractions to determine the distribution of the peroxisomes (catalase), mitochondrial matrix (glutamate dehydrogenase), mitochondrial inner membrane (cytochrome c oxidase), microsomes (esterase) and plasma membrane (alkaline phosphatase) in the gradient (figure 1). As shown, peroxisomes are well separated from the other organelles.

Phosphate uptake by proteoliposomes containing peroxisomal membrane protein
Increasing amounts of protein from highly purified peroxisomal fractions were reconstituted in proteoliposomes, and the time-dependent uptake of radiolabeled phosphate was followed in time (figure 2). As shown, a high rate of phosphate uptake was observed in proteoliposomes containing peroxisomal protein, whereas virtually no uptake was observed in liposomes without protein. The initial rate of phosphate uptake observed was approximately linear with protein. The phosphate uptake by the proteoliposomes approached a final level which was also linear with protein. Liposomes prepared with protein but without internal phosphate (the difference in osmolarity was compensated for by additional sucrose) exhibited a lower rate of phosphate uptake, amounting to about 70% of the activity in proteoliposomes containing phosphate. To investigate whether the transport of phosphate is influenced by a pH gradient across the liposomal membrane, as is the case with the mitochondrial phosphate transporter, we prepared proteoliposomes with either an internal pH of 7.5 or 5.5 and assayed the uptake of phosphate at different external pH values of 5.5 and 7.5. An initial rate of uptake of 67% ± 8% (pH_{in}>pH_{out}) and 63% ± 5% (pH_{out}>pH_{in}) was observed (n=2) relative to the control situation (pH_{in}=pH_{out}=6.5), indicating that a pH gradient has virtually no influence on the initial rate of uptake.

![Figure 3: The uptake of radiolabeled phosphate by proteoliposomes containing subfractionated peroxisomal protein. Soluble and membrane proteins were separated by sonication in high saline (1M NaCl), followed by high speed centrifugation (60 min at 100,000*g). The pellet and supernatant fractions were used to prepare proteoliposomes. In addition, proteoliposomes were prepared using total peroxisomal protein for comparison. The uptake of radiolabeled phosphate by each of these proteoliposomes is shown. The experiment was conducted in duplicate, the data shown are from a representative experiment.](image)
Figure 4: Activity of phosphate transport in different fractions obtained by Nycodenz gradient centrifugation of a light-mitochondrial fraction prepared from a fresh B. taurus kidney. Activity was measured as described in Materials and methods using the proteoliposome assay. The activity is expressed as a percentage of the total activity of all fractions. The rate of phosphate uptake by proteoliposomes prepared with each of the fractions was assayed in duplicate. The data shown are from a representative set.

To test the possibility that the observed protein-dependent phosphate uptake is caused by an increase in the internal liposomal volume as a consequence of the increasing concentrations of protein, sodium fluorescein was included in the vesicles during the reconstitution step. The internal volume was determined by measuring the fluorescence of enclosed fluorescein (excitation 494 nm / emission 518 nm) for the proteoliposome preparations with variable protein content and was found to be virtually constant. Small variations in the internal volume were not correlated to the protein content (table 1). This indicates that the presence of protein does not alter the liposomal volume. The intravesicular volume was found to amount to approximately 1.5 μl per mg of lipid.

To determine whether the protein responsible for the observed uptake is a membrane-bound or soluble protein, 20 μg of peroxisomal protein was further fractionated by sonication 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). Markers for the peroxisomal matrix (3-hydroxyacyl-CoA-dehydrogenase) and membrane (PMP70) were used to confirm the purity of both preparations (not shown). Subsequently, the soluble, membrane-associated and total peroxisomal protein fractions were reconstituted in proteoliposomes, and the phosphate uptake followed in time. As shown in figure 3, proteoliposomes containing peroxisomal membrane protein were found to take up phosphate at a much higher rate than proteoliposomes containing soluble protein.

Distribution of phosphate transport activity in different fractions obtained by Nycodenz gradient centrifugation

To determine the subcellular distribution of the phosphate transport activity observed in the proteoliposome assay, fractions 2 through 21 of the Nycodenz gradient shown in figure 1 were tested using this assay. For this purpose, the organelle pellets of all fractions were resuspended in identical volumes of reconstitution buffer and reconstituted in proteoliposomes. Radiolabeled phosphate uptake was determined after 10 minutes. Activity appears to be associated mainly with the peroxisomal.

### Table 1: Internal volume of proteoliposomal fractions containing variable amounts of protein, determined by measuring the fluorescence of enclosed sodium fluorescein. As shown, the internal volume is independent of the amount of peroxisomal protein used in the reconstitution procedure and amounts to approximately 1.5 μl per mg of lipid.

<table>
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<th>protein (μg)</th>
<th>internal volume (μl per mg lipid)</th>
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<tbody>
<tr>
<td>0</td>
<td>1.51</td>
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<tr>
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<tr>
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<td>1.47</td>
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<td>90</td>
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mitochondrial and plasma membrane fractions (figure 4).

**Differential sensitivity of phosphate transport activity in peroxisomal and mitochondrial fractions to p-hydroxy-phenylglyoxal and NEM**

In an attempt to differentiate between the activity found in peroxisomal and mitochondrial fractions, the effects of NEM (a sulfhydryl reagent) and p-hydroxy-phenylglyoxal (an arginine modifying reagent) were tested. Proteoliposomes were pre-incubated with 6.7 mM p-hydroxyphenylglyoxal or 3 mM NEM for 5 minutes at 35°C before starting the assay with radiolabeled phosphate. The amount of phosphate taken up was determined after 10 minutes. As shown in figure 5, the activity in the mitochondrial fraction (fraction 11 from the gradient in figure 1) is sensitive to both p-hydroxyphenylglyoxal and NEM and is reduced to approximately 50% by both inhibitors. In contrast, the activity associated with the peroxisomal fraction (fraction 4 from the gradient in figure 1) is not inhibited by these compounds, but is somewhat increased to approximately 135% of the control liposomes, by both inhibitors.

Previously, a higher sensitivity of the bovine mitochondrial phosphate transporter to sulfhydryl reagents such as NEM has been reported [27]. Therefore, higher concentrations of NEM were tested, but found not to further inhibit the mitochondrial activity (not shown). The mitochondrial peak fraction in the Nycodenz gradient also contains small amounts of other organelles, which might contribute to the remaining activity observed in the presence of inhibitors.

**Characterization of phosphate transport activity in peroxisomal fractions**

To establish some kinetic parameters of the peroxisomal phosphate transport activity, we determined the pH optimum and an approximate Km. The proteoliposome assay was performed at various pH values (equal internal and external pH) and phosphate uptake was determined after 10 minutes (figure 6A). Uptake was found to exhibit an optimum around pH=6.5. To determine a Km for phosphate uptake, increasing concentrations of phosphate were added, and the uptake followed in time. The rate of uptake as a function of the phosphate concentration is shown in figure 6B. However, Vmax was not reached, even at the highest concentration of phosphate, which prevented an accurate determination of the Km from these data. An approximate value of 39 mM was calculated by fitting the available data to the Michaelis-Menten equation. To test the possibility that the effect is caused by the increasing osmolarity of the external solution with phosphate concentration, a similar experiment was performed using sucrose solutions. Virtually no effect of sucrose was observed up to a concentration of 165 mM (not shown), which is iso-osmotic with 75 mM potassium phosphate solution [28].
Figure 6: Initial rate of phosphate uptake by proteoliposomes containing peroxisomal protein (A) as a function of the pH (pHin = pHout) and (B) as a function of the external phosphate concentration. Each point is the mean of duplicate measurements. The error bars indicate one standard deviation.

Direct measurement of the transport of pyrophosphate was not possible due to the unavailability of radiolabeled pyrophosphate. However, to obtain presumptive evidence that pyrophosphate is also a substrate for the observed phosphate transporter, increasing concentrations of unlabeled sodium pyrophosphate were added to the assay medium to compete with radiolabeled phosphate. The observed rate of phosphate uptake was decreased to approximately 50% of the maximal rate at a concentration of 42 mM pyrophosphate (figure 7).

Discussion

Our results demonstrate for the first time that the membrane from B. taurus kidney peroxisomes contains a transporter that facilitates the translocation of phosphate. The peroxisomal fractions of a Nycodenz gradient contain highly purified peroxisomes with no significant contamination from mitochondria or plasma membrane (figure 1). These fractions display phosphate transport activity in the proteoliposome assay employed, which is linear with protein and saturable. We did not observe an ‘overshoot’-phenomenon in our experiments, but this phenomenon is not always observed in counterflow-type experiments since it is caused by the combined effects of antiport activity and

Figure 7: Effect of increasing concentration of pyrophosphate on the rate of phosphate uptake by proteoliposomes containing peroxisomal protein. Proteoliposomes containing 20 μg of peroxisomal protein were used for this assay. Sodium pyrophosphate was added in the indicated amounts at the start of the assay. The data shown is the average and standard deviation of two experiments.
diffusion across the liposomal membrane. The charged, hydrophilic phosphate ion is not able to diffuse across a lipid bilayer at a significant rate, hence the leakage of imported radiolabeled phosphate is expected to be very low within the timeframe of our experiments. Indeed, the uptake of phosphate by liposomes without protein proceeds only at a very low rate, as shown in figure 2. Therefore, the amount of radiolabeled substrate taken up by the proteoliposomes is expected to approach isotopic equilibrium with time if every liposome contains at least one transporter molecule. The total intraliposomal volume can be calculated from the amount of intra-liposomal fluorescein, and was found to amount to approximately 1.5 µl per mg of lipid. From this, it may be estimated that when isotopic equilibrium is reached, the liposomes contain approximately 2700 DPM of radiolabeled phosphate in the experiment of figure 2. This is in excellent agreement with the observed value at high protein concentrations, when the concentration of liposomes without transporter molecules is lowest. Studies by other researchers also have shown that the overshoot phenomenon is not always observed in proteoliposomal assays of phosphate transport [29, 30].

The rate of uptake of radiolabeled phosphate was only slightly reduced in the absence of internal phosphate (figure 2), amounting to 69% of the control. Although this may suggest that uptake of phosphate is not obligatory coupled to the countertransport of another ion via the same carrier, future studies will have to reveal whether the transport of phosphate is truly unidirectional especially since it is well known that a single carrier may catalyze both an exchange reaction as well as unidirectional transport. A good example in this respect is the mitochondrial carnitine / acylcarnitine carrier which catalyzes the exchange between intramitochondrial (acyl)carnitine but also catalyzes the unidirectional uptake of carnitine [31].

Addition of pyrophosphate to the assay medium was found to reduce the uptake of phosphate. Although no direct evidence could be provided that pyrophosphate is transported due to the unavailability of radiolabeled substrate, it does suggest that pyrophosphate is able to compete with phosphate for uptake. An alternative explanation could be that the peroxisomal matrix contains a pyrophosphatase. However, no detectable pyrophosphatase activity was observed in the peroxisomal fractions of the nycodenz gradient (not shown).

The peroxisomal activity can be distinguished from the mitochondrial activity by its differential sensitivity to the inhibitors NEM and p-hydroxy-phenylglyoxal. Whereas the activity in the mitochondrial fraction is reduced by these reagents, the activity in the peroxisomal fraction is not. It should be noted that the activity of the yeast and human peroxisomal adenine nucleotide transporters Ant1p and PMP34 respectively, using ATP as substrate, were both found to be reduced by NEM [16].

The assay method employed in this paper, in which peroxisomal membrane proteins are reconstituted in liposomes, enables direct measurement of transport processes that are not possible with isolated peroxisomes due to the permeability of the membrane of isolated peroxisomes. Generally, reconstitution requires relatively large amounts of protein, which is usually acquired by overexpression of the protein in a suitable host. However, to investigate transport processes mediated by proteins for which no gene has previously been identified, as in the case of the phosphate transporter described here, overexpression cannot be employed. In this report we show that overexpression is not strictly required to enable the measurement of peroxisomal transporters. This method may be employed to identify transporters for other peroxisomal metabolites in the future.

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References

Pyrophosphate transport

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