The rat liver peroxisomal membrane forms a permeability barrier for cofactors but not for small metabolites in vitro

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Summary

The functional role of the peroxisomal membrane as a permeability barrier to metabolites has been a matter of controversy for more than four decades. The initial conception, claiming free permeability of the membrane to small solute molecules, has recently been challenged by several observations suggesting that the peroxisomal membrane forms a closed compartment. We have characterized in vitro the permeability of rat liver peroxisomal membrane. Our results indicate that the membrane allows free access into peroxisomes for small hydrophilic molecules, such as substrates for peroxisomal enzymes (glycolate, urate), but not to more bulky cofactors (NAD/H, NADP/H, CoA). Although access for cofactors is not prevented completely by the membrane, the membrane barrier severely restricts their rate of entry into peroxisomes. The data lead to conclusion that, in vivo, peroxisomes may possess their own pool of cofactors, while they share a common pool of small metabolites with the cytoplasm. The results also indicate that molecular size plays an important role in in vivo distinction between cofactors and metabolic intermediates.

Key words: Peroxisomes, Membrane permeability, Cofactors

Introduction

Peroxisomes are nearly ubiquitous in eukaryotic cells. These subcellular organelles harbor over 60 proteins that participate in different metabolic pathways and their vital functional importance is exemplified by the existence of inherited metabolic disorders with severe consequences (Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum's disease) determined by malfunctioning peroxisomes (reviewed in Masters and Crane, 1995; Mannaerts et al., 2000; Wanders et al., 2001; Hiltunen et al., 2003; Van Roermund et al., 2003).

The functional role of the peroxisomal membrane as a permeability barrier to substrates and cofactors has been discussed for more than 40 years. There are currently two contrasting viewpoints: (1) the peroxisomal membrane is freely permeable in vivo to water-soluble molecules with molecular masses up to 800-1000 Da, including cofactors (NAD/H, NADP/H, CoA,); (2) the peroxisomal membrane is completely impermeable in vivo to small hydrophilic molecules and accessibility of peroxisomes to these substances under in vitro conditions is owing to a loss of peroxisomal integrity during their isolation.

The first viewpoint emerged in the course of early studies on rat liver peroxisomes in an attempt to explain: (a) the unusual behavior of these organelles upon equilibrium-density centrifugation in sucrose gradients, which implies that peroxisomes are readily permeable to sucrose; (b) the finding that, in contrast to mitochondrial and lysosomal enzymes, the peroxisomal oxidases known at that time (urate oxidase, D-amino acid oxidase and L-α-hydroxyacid oxidase) were unable to show structure-linked latency (De Duve and Baudhuin, 1966; Leighton et al., 1968; Baudhuin, 1969). Direct assessment of the incorporation of some radioactive substances (carnitine, sucrose, NAD^+, ATP, CoA) into isolated rat liver peroxisomes and permeability measurements to these solutes using liposomes reconstituted with peroxisomal membrane proteins, led to proposal that the membrane of peroxisomes – similar to outer membranes of mitochondria and chloroplasts – contains porins i.e. channel-forming proteins (Van Veldhoven et al., 1983; Van Veldhoven et al., 1987). A patch-clamp technique using purified peroxisomal membrane fragments incorporated into liposomes (Lemmens et al., 1989) or fusion of peroxisomal membrane to planar lipid bilayers (Labarca et al., 1986) revealed the presence of a weakly cation-selective large conductance channel with an estimated diameter of 1.5-3.0 nm. More recent evidence in favor of the presence of peroxisomal membrane porins include: (a) the observation that several peroxisomal oxidases (urate oxidase, L-α-hydroxyacid oxidase and D-amino acid oxidase) possess no latency in digitonin-permeabilized rat hepatocytes, whereas this type of treatment (in contrast to e.g. tissue homogenization) is much more protective for subcellular organelles, especially peroxisomes (Verleur and Wanders, 1993); (b) the evidence on the presence of pore-forming proteins in peroxisomes (glyoxysomes) from plants (Reumann, 2000) and yeasts (Sulter et al., 1993); (c) the conclusion, based on experiments with intact cells, that the mammalian peroxisomal membrane is highly permeable to hydrogen ions (H^+) and that the

intraperoxisomal pH is the same as that in the surrounding cytoplasm (Jankowski et al., 2001).

The initial data conflicting with the 'free-permeability' concept came from in vitro experiments showing that, in contrast to 'classical' peroxisomal oxidases (urate oxidase, D-amino acid oxidase and L-α-hydroxyacid oxidase), some other enzymes confined to these particles such glucose-6-phosphate dehydrogenase (Antonenkov, 1989) and acyl-CoA:dihydroxyacetonephosphate acyltransferase (Wolvetang et al., 1990), possess structure-linked latency. Studies exploiting genetic approaches, notably in the yeast *Saccharomyces cerevisiae*, showed the vital importance of shuttle mechanisms for the proper functioning of peroxisomal metabolic pathways in lower eukaryotes (Van Roemund et al., 1995; Van Roemund et al., 1998; Van Roemund et al., 1999; Kal et al., 1999). Several enzymatic activities have been observed (lactate dehydrogenase, glucose-6-phosphate dehydrogenase, carnitine acyltransferases) that may represent the intraperoxisomal part of the shuttle systems for mammalian peroxisomes (Antonenkov, 1989; Masters and Crane, 1995; Baumgart et al., 1996; Wanders et al., 2001). The presence of metabolic shuttles is considered as a proof that peroxisomes (at least under in vivo conditions) form a closed compartment impermeable to low-molecular-mass solutes. This concept has been supported recently by revealing solute carriers related to the mitochondrial transporter superfamily in yeast (Palmiery et al., 2001; Van Roemund et al., 2001) and mammalian peroxisomes (Weber et al., 1997; Wylin et al., 1998; Visser et al., 2002). Finally, in situ determination of pH, by targeting a pH-sensitive fluorescent reporter group to peroxisomes, suggested the existence of a pH gradient across the membrane of these organelles in living fibroblasts (Dansen et al., 2000).

Here, we present experimental results indicating that the rat liver peroxisomal membrane is freely permeable to small metabolites but bulky cofactors, as other large molecules, are prevented from free movement into and out of peroxisomes. A possible functional role of this basic principle of peroxisomal membrane physiology is discussed.

Materials and Methods

Materials

Nycodenz [5-(N-2,3-dihydroxypropylacetamido)-2,4,6-triiodo-N,N′-bis(2,3-dihydroxypropyl)-isophthalamide], sucrose and other sugars, polyethylene glycols (PEGs), proteinase K, phenylmethanesulfonyl fluoride (PMSF) and bovine serum albumin were obtained from Sigma (St Louis, MO). Percoll (colloidal suspension of silica), 2′5′-ADP-Sepharose 4B, 5′-AMP-Sepharose 4B and Blue-Sepharose were from Amersham Biosciences (Uppsala, Sweden). Fixatives and other reagents for electron microscopy were from Electron Microscopy Sciences (Fort Washington, PA). Other chemicals used were of pro-analysis grade.

Isolation and enzyme content of purified peroxisomes

Male Sprague-Dawley rats weighing 200-250 g were used after overnight starvation. Their livers were homogenized (1:4 w/v) in isolation-medium 1 [0.25 M sucrose, 10 mM MOPS buffer pH 7.4, 1 mM EDTA, 1 mM EGTA, 2 mM dithiotreitol (DTT), 0.1 mM PMSF]. A light mitochondrial fraction most enriched in lysosomes and peroxisomes was isolated by differential centrifugation. This fraction was then subjected to isopycnic centrifugation in a self-generating Percoll gradient (Antonenkov et al., 1997). Fractions enriched in peroxisomes (from the top of gradient) were collected and loaded on multistep Nycodenz gradients [the volume of each gradient was 32 ml, they consist of 16-50% w/v Nycodenz solutions prepared in isolation-medium 2 (isolation medium 1 without sucrose)] that were kept overnight at 4°C. The samples were centrifuged in a vertical rotor at 100,000 $\boldsymbol{g}_{max}$ for 90 minutes applying slow acceleration and deceleration mode. Purified peroxisomes were diluted with isolation-medium 2 and centrifuged at 40,000 $\boldsymbol{g}_{max}$ for 30 minutes to remove soluble proteins that had escaped from broken particles. The recovery of soluble matrix proteins in the pellet was 70-80% for catalase and 65-70% for other enzymes tested (L-α-hydroxyacid oxidase, lactate dehydrogenase and glucose-6-phosphate dehydrogenase). The pellet was resuspended in isolation-medium 2 and used immediately for the determination of the latency of peroxisomal enzymes and also in other experiments described below. To minimize potential oxidative damage to membrane components such as polyunsaturated fatty acids or proteins, we used only freshly isolated peroxisomal preparations. According to our observations, the membrane of peroxisomes may suffer transient rupture during isolation of the particles. However, like other membrane structures such as erythrocytes or the endoplasmic reticulum, it seals after disruption, restoring its permeability properties (V.D.A. et al., unpublished). This behavior of peroxisomes explains some leakage of matrix proteins from the particles (see above).

To investigate the peroxisomal localization of some enzymes that have been reported to be present in these particles, we compared the pattern of their distribution in the Nycodenz gradient with that of markers for different organelles: peroxisomes (catalase, L-α-hydroxyacid oxidase), mitochondria (glutamate dehydrogenase), endoplasmic reticulum (esterase) and lysosomes (acid phosphatase). The activity of some enzymes tested in purified peroxisomal preparations was low (less than 2-3% of the total activity in the homogenate) implying the possibility of their nonspecific adsorption on the outer surface of the particles. To further verify the localization of these enzymes inside peroxisomes, we treated freshly isolated organelles with proteinase K. We also reinvestigated the intraperoxisomal localization of some enzymes to verify their presence in the matrix as soluble constituents. After disruption of peroxisomes by sonication (Alexson et al., 1985), we separated the particles from soluble proteins by equilibrium-density centrifugation in a multistep sucrose gradient (Antonenkov, 1989).

Assay of enzymes and latency determination

The protein content and the activities of marker enzymes for subcellular organelles and different peroxisomal enzymes were determined according to standard procedures (for deatails, see Leighton et al., 1968; Fujiki et al., 1982; Antonenkov, 1989; Antonenkov et al., 1997). D-Amino acid oxidase and glycerol-3-phosphate dehydrogenase activities were measured with D-alanine and dihydroxyacetone phosphate as the substrates, respectively. The activity of peroxisomal 3-oxoacyl-CoA thiolase was determined in the acetoacetyl-CoA cleavage assay at 306 nm (ε: 3600 M^{-1} cm^{-1}). The activity of xanthine oxidizing enzyme was measured with 0.6 mM NAD^+ (dehydrogenase form) or without (oxidase form) by monitoring uric acid accumulation at 292 nm after urate oxidase had been inhibited by oxonate (Hashimoto, 1974). Units of enzyme activity are given as μmol of substrate consumed or product produced per minute. Catalase activity is expressed in units defined elsewhere (Alexson et al., 1985).

Latency of the enzymes confined to peroxisomes was determined by comparing their 'free' and 'total' activities as described for catalase (Baudhuin, 1969). Free activity was determined at 25°C in the standard assay mixture for the enzyme activity determination. After recording this activity, Triton X-100 (0.05% w/v, final concentration) was added to determine the total activity. Separate experiments showed that adding 0.25 M sucrose (as a potential osmoprotector) to the assay mixture did not affect latency of peroxisomal enzymes.

To determine latency of peroxisomal enzymes, we initially used

fractions obtained directly from a Nycodenz gradient which have been diluted with isolation-medium 2. The results revealed high levels of free activity for peroxisomal enzymes possessing latency (up to 30-50% of the total activity). A substantial fall in the free activity was found when soluble proteins escaping from the particles were removed by resedimentation of purified peroxisomes were removed. Exceptions to this were elevated levels of free activities for lactate and glycerol-3-phosphate dehydrogenases because of their high nonspecific ionic interaction with biological membranes (V.D.A., R.T.S. and J.K.H., unpublished results). Therefore, in some experiments the particles were resedimented in the presence of 0.15 M KCl (final concentration).

Swelling assay

Swelling experiments were performed at 25°C using a Shimadzu UV-3000 spectrophotometer (Shimadzu, Kyoto, Japan) as described previously for liposomes (Blachly-Dyson et al., 1997) with some modifications. The level of turbidity of peroxisomal suspension [measurements of optical density (OD) at 520 nm] was used as an indicator of particles swelling (decrease in OD because of higher transparency of the particles) or shrinking (increase in OD because of higher scattering of the light by condensed peroxisomes). Freshly prepared peroxisomes were resedimented (to remove Nycodenz) and resuspended in isolation-medium 2 at a final protein concentration of 8-10 mg/ml. This suspension was diluted 15-fold with isolation-medium 2 (25°C) immediately before the swelling assays, and the OD of peroxisomes was measured at 520 nm against isolation-medium only. Because separate experiments showed that peroxisomes, in contrast to e.g. liposomes, do not sediment in the cuvette, swelling assays were performed without stirring.

Intraperoxisomal NADH and NADPH absorbance

This experiment is based on the assumption that cofactors slowly penetrate into peroxisomes and reach a concentration equilibrium inside and outside the particles during a certain period of time if a membrane barrier is present. Under these conditions, the sudden oxidation of NADPH outside peroxisomes would not lead to its simultaneous oxidation inside the particles. The absorbance of intraperoxisomal cofactor can be registered and compared with the data obtained after disruption of the membrane by detergent. To oxidize NADPH, we used a mixture containing glutamate dehydrogenase and its substrates that is similar to the one previously exploited in cyclic enzymatic assays (Passonneau and Lowry, 1974).

Latency of peroxisomal lactate dehydrogenase

A high peroxisomal concentration of an enzyme may lead to conditions where its activity is greater than the rate of transmembrane transfer of the substrate (Baudhuin, 1969; Poole, 1975) (see also Results). Two tests have been described to verify this mechanism (Baudhuin, 1969). We exploited these tests to confirm our proposal that the latency of peroxisomal cofactor-dependent enzymes is determined by the slow diffusion rate of cofactor through the membrane by studying peroxisomal lactate dehydrogenase activity (see Results). Lactate dehydrogenase is a soluble matrix protein that escapes easily from broken peroxisomes. Therefore, in our preliminary experiments, we tried to discriminate which portion of free lactate dehydrogenase activity is determined by the rate of cofactor diffusion through the membrane (i.e. real free activity) relative to activity caused by the enzyme escaped from peroxisomes. Attempts to remove soluble lactate dehydrogenase by resedimentation of peroxisomes failed, probably owing to persistent damage of some particles. Treatment of peroxisomes by proteinase K was not acceptable because of its effect on peroxisomal membrane permeability (see Results). The desired result was obtained by incubating purified peroxisomes with affinity-media, which binds NAD(P)-dependent dehydrogenases (5′-AMP-Sepharose, Blue-Sepharose, 2′,5′-ADP-Sepharose). In the final procedure, the purified peroxisomes (4-5 mg protein/ml) were resedimented in the presence of 0.15 M KCl, suspended in 20 mM MOPS pH 7.4, and mixed for 40 minutes at 4°C with an equal volume of 2′,5′ADP-Sepharose (50 mg/ml) equilibrated with the same buffer. Affinity beads were removed from the mixture by low-speed centrifugation. This treatment results in the complete removal of unsedimentable lactate dehydrogenase from the peroxisomal suspension.

Stopped-flow experiments

Peroxisomes were exposed to an osmotic gradient by rapid mixing with an equal volume of isolation-medium 2 containing PEGs. Experiments were performed using a rapid mixing attachment (model RMA-1A) to a Shimadzu UV-3000 spectrophotometer according to the manufacturer's instruction.

Electron microscopy

A suspension of peroxisomes in isolation-medium 2 (0.4-0.6 mg protein/ml) was fixed with 2% glutaraldehyde overnight at 4°C and sedimented at 20,000 g_{max} for 30 minutes. The pellets were stained with 1% OsO_4 in 0.1 M PIPES (pH 7.4) for 1 hour and then incubated with 1% uranyl acetate for 1 hour. The samples were dehydrated and embedded in Epoxy-embedding-medium according to the manufacturer's instruction (Fluka, Buchs, Switzerland). Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips EM 410 microscope.

Results

Purity and enzyme content of rat liver peroxisomal fraction

This investigation relies on the use of a peroxisomal fraction without mixture of other subcellular particles. The composition of the peroxisomal preparation was estimated from the specific activities of the marker enzymes (Table 1). The peroxisomes were purified about 37-fold over the original homogenate. The purified fraction constitutes approximately 7% of the total peroxisomal enzyme activity and 0.18% of the protein content of the whole liver, which is in accordance with the results described for highly purified peroxisomal preparations by others (Leighton et al., 1968; Fujiki et al., 1982; Alexson et al., 1985; Baumgart et al., 1996). Electron microscopic examination of the preparation revealed a nearly homogeneous population of peroxisomes (Fig. 1).

To study latency of peroxisomal enzymes (see below), several of them were selected according to the following criteria: (a) enzymes that are present inside the particles; (b) enzymes that are soluble matrix proteins; (c) enzymes that catalyze reactions with water-soluble substrates and (d) enzymes that rely on soluble cofactors such as NAD/H, NAD(P)/H or CoA for their activity. To evaluate compliance with these criteria, we reinvestigated the subcellular distribution of selected cofactor-dependent enzymes that were described previously as peroxisomal constituents in mammals and examined their intraperoxisomal localization in certain cases. Our data confirmed previous results (reviewed in Master and Crane, 1995; Mannaerts et al., 2000) showing that rat liver peroxisomes contain in their matrix the following cofactor-dependent enzymes in a soluble form: lactate dehydrogenase, NADH; glycerol-3-phosphate dehydrogenase, NADH; glucose-6-phosphate dehydrogenase, NADPH; isocitrate dehydrogenase, NADPH; and the majority (over 70% of the total peroxisomal

Table 1. Properties of purified peroxisomal fractions

Enzyme (protein)	Absolute activity in homogenate (U/g liver)	Composition of fractions (% of activity in homogenate)			Relative specific activity in purified peroxisomes with respect to whole liver
		Light mitochondrial fraction	Fraction after Percoll gradient	Purified peroxisomal fraction*	
Protein (mg/g liver)	202.4±16.2	6.6±1.2	2.8±0.6	0.18±0.07	1.0
Catalase	51.2±4.6	26.6±5.8	18.7±4.2	6.6±0.8	36.8
Urate oxidase†	3.6±0.9	41.6±9.3	23.8±6.3	7.2±0.9	40.0
Glutamate dehydrogenase	10.4±2.5	29.0±6.7	10.7±2.8	0.03±0.01	0.16
Esterase	366.0±28.5	13.3±2.7	7.6±2.1	0.03±0.01	0.16
Acid phosphatase	225.4±25.2	10.2±2.4	4.1±1.2	0.04±0.02	0.21

*Enzyme activity was measured before resedimentation of the particles (see Materials and Methods).
†Activity of urate oxidase was measured after resedimentation of peroxisomes to avoid interference of the Nicodenz UV absorbance on the activity determination.
Values are given as the means±s.d. (n=3-4).

activity) of octanoyl-CoA:carnitine acyltransferase activity. Peroxisomal acetyl-CoA:carnitine acyltransferase is present mainly in the membrane of normal rat liver peroxisomes.

Latency of peroxisomal dehydrogenases and CoA-dependent enzymes

The latency of enzymes confined to different organelles such as mitochondria or lysosomes, is a well-known phenomenon reflecting the existence of a membrane barrier to substrates. Determination of the latency of peroxisomal oxidases, NADH- and NADPH-dependent dehydrogenases and CoA-dependent enzymes under identical conditions in a highly purified peroxisomal fraction revealed that, in contrast to the oxidases, other peroxisomal enzymes tested, showed latent activities (Table 2). Interestingly, the latency phenomenon was shown only for peroxisomal enzymes that rely in their activity on the presence of soluble cofactors (NAD/H, NAD(P)/H or CoA) in the incubation media [with catalase being an exception (see Baudhuin, 1969; Poole, 1975)]. To further study this observation, we exploited the ability of the peroxisomal xanthine-oxidizing enzyme to use NAD^+ (xanthine dehydrogenase form) or oxygen (xanthine oxidase form) as an electron acceptor (reviewed in Harrison, 2002). The enzyme is bound to a crystalline structure (Angermuller et al., 1987) formed by urate oxidase inside peroxisomes that is called a nucleoid (Masters and Crane, 1995). In a freshly prepared peroxisomal fraction the xanthine-oxidizing enzyme shows mainly xanthine dehydrogenase activity (Fig. 2A). This dehydrogenase form (cofactor-dependent) revealed a high level of latency, whereas treatment with detergents or sonication did not affect the activity of the xanthine oxidase form (cofactor-independent), indicating complete absence of latency.

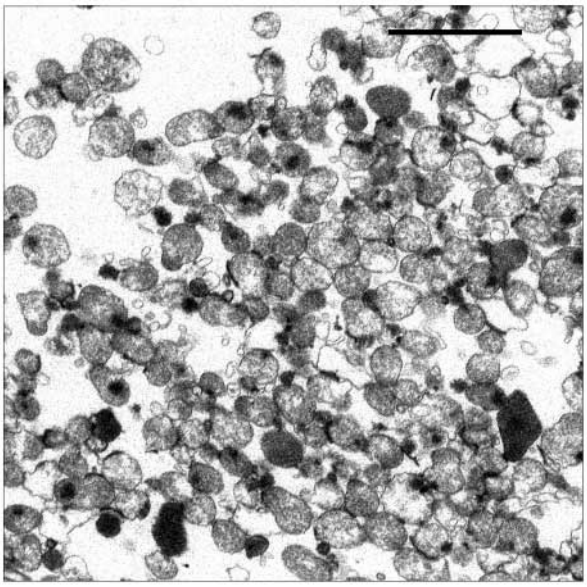

Fig. 1. Purity of rat liver peroxisomal fraction. Electron micrograph of purified peroxisomes from the Nycodenz gradient. Scale bar, 2 μm.

These data lead to the hypothesis that the peroxisomal membrane is permeable under in vitro conditions to water-soluble substrates for intraperoxisomal enzymes, but not to soluble cofactors. The simplest explanation to this phenomenon emerges when the molecular size of common metabolites (usually less that 200 Da) and cofactors (600–800 Da) is taken into consideration.

Factors affecting latency of peroxisomal enzymes

In our preliminary experiments, we tested various treatments that cause damage to the biological membrane (the detergents Triton X-100 and deoxycholate, sonication, and freezing and thawing) in an attempt to uncover the latent activity of peroxisomal enzymes. The best results were obtained with Triton X-100. The effect of detergent implies an involvement of membrane lipids in maintaining latency of peroxisomal enzymes. This was confirmed when we prepared an incubation of isolated peroxisomes with phospholipase C in the presence of Ca^{2+}. As expected, a gradual increase in free activity of lactate dehydrogenase (Fig. 2B), glucose-6-phosphate dehydrogenase and 3-oxoacyl-CoA thiolase (data not shown) was observed. Resedimentation of peroxisomes treated with detergents or phospholipase C showed that the bulk of free activity is owing to the release of the enzymes from the particles.

Inactivation of the membrane's protein component revealed an unexpected result – a significant reduction in the peroxisomal membrane permeability, not only to cofactors but also to small metabolites such as substrates for peroxisomal oxidases. For instance, thermal treatment gradually inactivated the enzymes confined to peroxisomes (see legend to Fig. 2C). This expected phenomenon was accompanied by the appearance of the latent activities of urate oxidase and L-α-hydroxyacid oxidase, which were clearly detectable even before the decrease in the total activities of these enzymes (Fig. 2C). Similarly, treatment with

Table 2. Specific activity and latency of the enzymes in purified peroxisomal fraction*

Enzyme	Cofactor (or derivative) in the incubation medium	Specific activity (U/mg protein)	Free activity (% of total activity)
Catalase		5.3±0.2	22.4±0.9
Urate oxidase		0.88±0.06	94.5±3.8
L-α-hydroxyacid oxidase		0.25±0.04	93.6±3.2
D-amino acid oxidase		0.14±0.03	95.4±4.0
Lactate dehydrogenase†	NADH	0.97±0.12	20.7±2.1
Glycerol-3-phosphate dehydrogenase†,‡	NADH	0.27±0.09	22.0±3.0
Isocitrate dehydrogenase	$NADP^+$	0.20±0.06	19.8±2.7
Glucose-6-phosphate dehydrogenase	$NADP^+$	0.12±0.03	18.4±2.2
3-Oxoacyl-CoA thiolase	CoA (acetoacetyl-CoA)	0.42±0.05	18.6±3.0
Octanoyl-CoA:carnitine acyltransferase	Octanoyl-CoA	0.10±0.03	17.2±2.8

*Enzyme activities were determined after resedimentation of peroxisomes from Nycodenz gradient (see Materials and Methods).
†'Free' activity was measured after resedimentation of the particles in the presence of 0.15 M KCl.
‡'Total' activity was determined after sonication of the particles due to inhibitory effect of Triton X-100 on the enzyme activity. Values are given as the means±s.d. (*n*=4-5).

protease did not significantly change the total activity of the enzymes tested unless the peroxisomal membrane was disrupted by detergent (Fig. 2D, left panel). However, the effect of protease on the free activity of the enzymes was profound (Fig. 2D, right panel). The free activities of catalase and lactate dehydrogenase rapidly declined to near zero. Moreover, the activities of urate oxidase and L-α-hydroxyacid oxidase became increasingly latent. The appearance of latency of peroxisomal oxidases after thermal and protease treatments challenges the wide-spread supposition claiming the loss of integrity of the peroxisomal membrane upon isolation of the particles.

Size-exclusion limit for peroxisomal membrane permeability

The latency experiments indicate that the size-exclusion limit for the permeability of the peroxisomal membrane can be expected to be between the sizes of common metabolites and cofactors, i.e. 400-500 Da. However, this is difficult to reconcile with the results reported previously, which indicate a much larger pore size for the peroxisomal channel(s) (see Introduction). To resolve this contradiction, we designed experiments to assess the size-exclusion limit for peroxisomal membrane permeability.

The latency of cofactor-dependent enzymes indicates that the peroxisomal membrane forms a restricting barrier to at least some (bulky) organic solutes. This may provoke the behavior of peroxisomes as true osmometers (Alberts et al., 2002). Hydrophilic compounds that are unable to permeate the membrane of peroxisomes can probably serve as osmoprotectors for these particles. To verify this assumption, we chose to examine the effects of commercially available PEGs on peroxisome integrity. Osmotic damage to peroxisomes was determined by measuring the activity of soluble matrix enzymes (catalase and L-α-hydroxyacid oxidase) that leaked out of broken particles. PEGs with a molecular mass of 1000 Da or larger provide an effective protection to peroxisomes during homogenization (Fig. 3A) indicating that these molecules do not penetrate into the particles. The size of about 1000 Da agrees with the previously reported size-exclusion limit (see Introduction). However, it is larger than the dimensions of the cofactors. This limit does not restrict the permeability of Nycodenz (821 Da) which we used for the isolation of peroxisomes. Accessibility of the particles to sucrose (342 Da), Nycodenz or even Optiprep (1550 Da) is a generally accepted explanation for the unusually high equilibrium density of peroxisomes during centrifugation in gradients formed by these media (reviewed in De Duve and Baudhuin, 1966; Masters and Crane, 1995). Dilution of sucrose gradient medium [as well as Nycodenz medium (V.D.A., R.T.S. and J.K.H., unpublished results)] that contains peroxisomes by solutions with much lower tonicity leads to a partial destruction of the particles owing to temporal osmotic disbalance (Baudhuin, 1969). The application of PEGs to prevent this damage revealed that, like in the case of tissue homogenization, the limit of permeability for the peroxisomal membrane is about 1000 Da (data not shown). In both cases, molecules with a size of less than 400 Da show a very low protective effect (see Fig. 3A) explaining the inefficiency of sucrose as an osmoprotector of peroxisomes (Baudhuin, 1969; Masters and Crane, 1995).

To further study the peroxisomal size-exclusion limit, we designed bottle-stopper experiments as an additional approach (Fig. 3B). When the latency of urate oxidase was taken as an indicator for the closure of the hypothetical peroxisomal channel by PEG molecules, the lowest free activity was found in the presence of PEG 1000 (Fig. 3C).

The peroxisomal size-exclusion limit was also verified by so-called swelling experiments (reviewed in Benz and Bauer, 1988; Zamzami et al., 2001). For this purpose, we used two chemically different groups of uncharged compounds, PEGs and sugars, and assessed their ability to penetrate into peroxisomes. At first we compared the turbidity of a peroxisomal suspension in samples containing sugars over a control without any solutes. The resulting curve showed a direct dependence of the OD at 520 nm on the molecular size of the sugars. The data show that the turbidity reaches a plateau when samples contain sugars with molecular masses of more than 900-1000 Da, indicating this level as a size-exclusion limit for peroxisomal membrane permeability (data not shown). During measurements of peroxisomal turbidity, we observed a drift towards lower optical density that lasted far beyond the detection time (1 minute) after the addition of certain sugars. This feature was not observed with control samples (without sugar). We performed a more detailed analysis of this phenomenon by using PEGs of different molecular masses. Mixing peroxisomes with PEG-free buffer (control) led to an abrupt fall in OD within one minute without further change in OD value (Fig. 3D, left panel). A similar abrupt fall in OD without a drift towards lower OD values was also

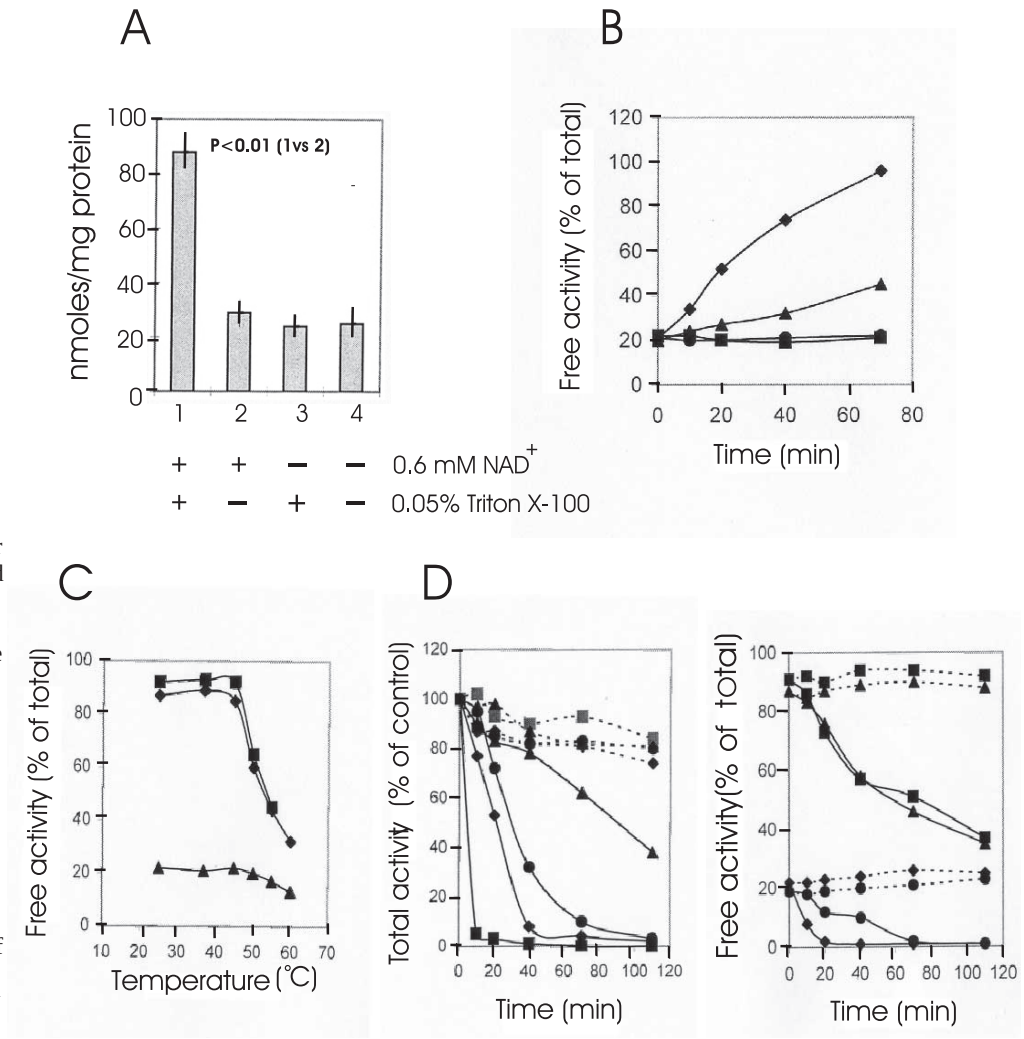

Fig. 2. Latency of peroxisomal enzymes. (A) Activity of xanthine-oxidizing enzyme in a purified peroxisomal fraction. Values given are means±s.d. (*n*=4-5). (B) Effect of phospholipase C on the free activity of lactate dehydrogenase. Aliquots of purified peroxisomes in 20 mM MOPS, pH 7.4 (0.4 mg protein/ml) were incubated at 37°C with 3 mM $CaCl_2$ (▲), 1 U/ml phospholipase C (●), $CaCl_2$ with phospholipase C (◆) or without any additions (■). The reaction was interrupted by 10 mM EDTA (final concentration). Panels B-D show the free activity relative to 100% of the total activity in the same sample. (C) Thermal treatment of purified peroxisomes. Aliquots of peroxisomes in isolation-medium 2 (0.4 mg protein/ml) were incubated for 3 minutes at different temperatures and then diluted with 5 volumes of the same ice-cold medium. The free activities of catalase (▲), urate oxidase (◆) and L-α-hydroxyacid oxidase (■) were immediately determined. The total activity of the oxidases after thermal treatment of peroxisomal suspension (relative to activity without treatment) at 37°C, 45°C, 50°C, 55°C and 60°C were: 100%, 104%, 98%, 66% and 43% for urate oxidase and 97%, 98%, 94%, 86% and 52% for L-α-hydroxyacid oxidase, respectively. Notice the appearance of the latency of urate oxidase and L-α-hydroxyacid oxidase at 50°C while the total activity of the enzymes was not suppressed at this temperature. (D) Proteinase K treatment of purified peroxisomes. Aliquots of the particles in 20 mM MOPS, pH 7.4 (0.4 mg protein/ml) were incubated in the presence of 0.6 U/ml proteinase K at 37°C. The reaction was stopped by the addition of 1 mM PMSF (final concentration). The left panel shows the activity of the enzymes in the samples incubated with proteinase K and 0.05% (w/v) Triton X-100 (solid line). In the parallel samples the detergent was added after the incubation with proteinase K (hatched line). Data are presented as a percentage of activity relative to the control (without incubation). The right panel displays the free activity of enzymes in the samples incubated with (solid line) or without (hatched line) proteinase K. Catalase,●; urate oxidase, ▲; L-α-hydroxyacid oxidase, ■; lactate dehydrogenase,◆.

observed after mixing peroxisomes with PEG 200 and PEG 1500, whereas the other PEGs tested (Fig. 3D, right panel) provoked prolonged swelling of the particles during the registration period (10 minutes). Fig. 3E summarizes the dependence of relative ODs on the size of PEGs after the incubation of peroxisomes for 1 minute and 10 minutes. Within this time, the most prominent difference in OD values was noticed for PEG 600, demonstrating its slow uptake into peroxisomes.

Cofactors slowly penetrate into peroxisomes

Data described in the previous section indicate that the size-exclusion limit for the peroxisomal membrane is higher than the size of cofactors such as NADH (663.4 Da), $NADP^+$ (743.4 Da) or CoA (767.5 Da). This implies that cofactors may penetrate the peroxisomal membrane. Our latency experiments, however, support the view that the membrane of peroxisomes is impermeable to cofactors. To resolve this contradiction, we designed experiments to directly assess peroxisomal membrane permeability to cofactors. The effect of NAD^+ and $NADP^+$ on the osmotic behavior of purified peroxisomes clearly indicates the slow uptake of the cofactors into peroxisomes (Fig. 4A). In another approach, we exploited the intraperoxisomal NADH or NADPH absorbance (Fig. 4B). This experiment offers the possibility to estimate the permeability to cofactors at near physiological concentrations (0.20-0.25 mM). Prolonged incubation of purified peroxisomes with NADPH followed by its abrupt enzymatic oxidation led to the appearance of an absorbance peak at 340 nm. This peak instantly disappeared after the addition of detergent. However, in the absence of detergent there was a gradual decrease in absorbance, possibly implying slow diffusion of NADPH out of peroxisomes. Although we were unable to precisely measure the rate of this reverse diffusion, the indications were that the equilibrium of NADPH inside and outside peroxisomes is a slow process, accomplished within about 20 minutes incubation time. Similar results were obtained by using NADH (data not shown).

The low diffusion rate for cofactors into peroxisomes may cause latency of cofactor-dependent peroxisomal enzymes. The mechanism of this phenomenon may be similar to the latency of peroxisomal catalase (Baudhuin, 1969; Poole, 1975). We used two tests, analogous to those described earlier for catalase (Baudhuin, 1969), to confirm this assumption. We investigated (a) the effect of intraperoxisomal lactate dehydrogenase inhibition on the latency of this enzyme and (b) the effect of NADH concentration on free lactate dehydrogenase activity. Inhibition of intraperoxisomal lactate dehydrogenase by oxamate is accompanied by an increase in the free enzyme activity (Fig. 4C). However, this effect is evident only after severe suppression of the total enzyme activity (over 100 times, relative to the control without inhibitor) indicating an enormous prevalence of the rate of cofactor conversion in peroxisomes over the level of its diffusion into the particles. The second test shows

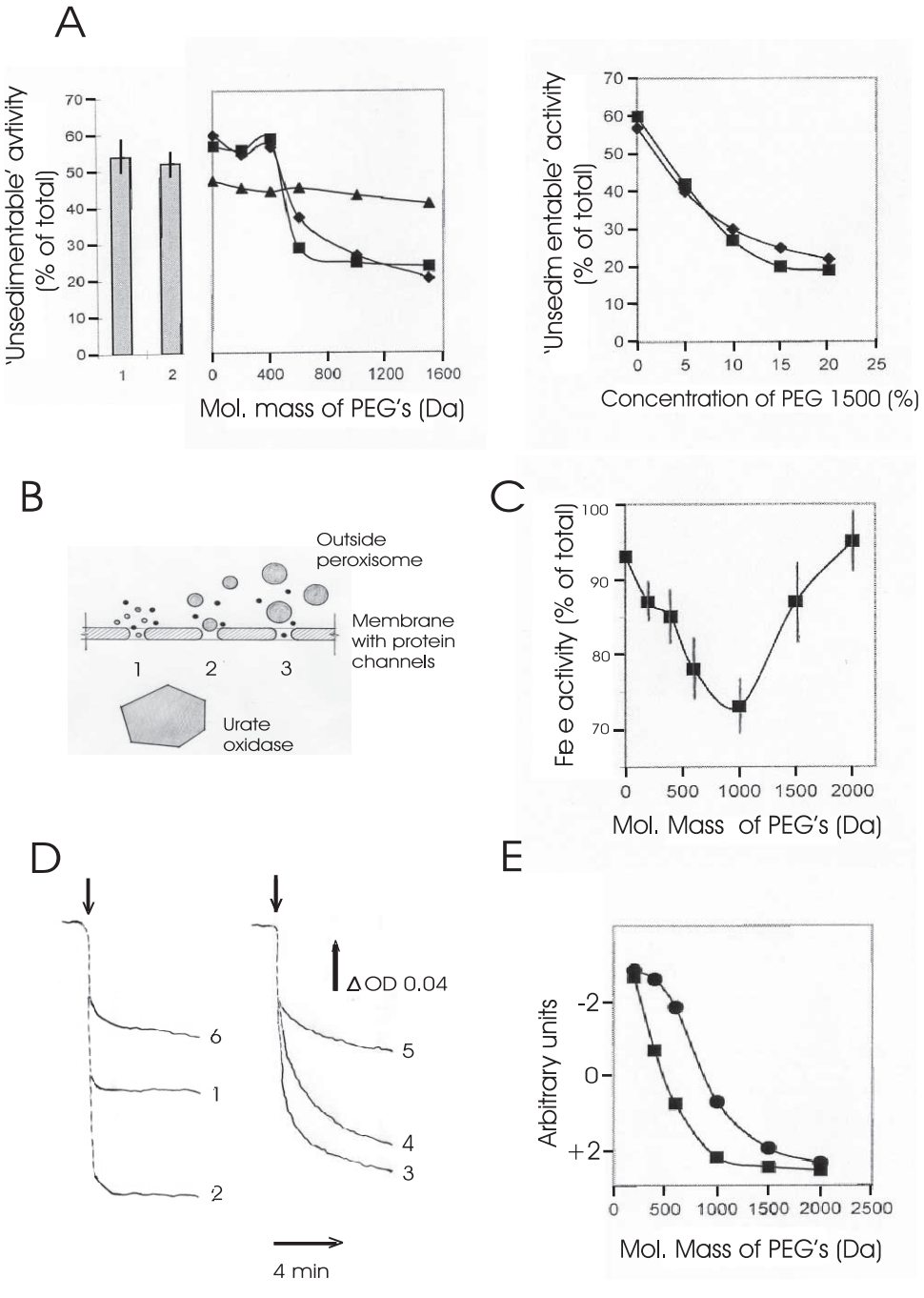

Fig. 3. Size-exclusion limit for peroxisomal membrane permeability. (A) Effect of PEGs on solubilization of enzymes from peroxisomes during rat liver homogenization. Livers were perfused to wash out erythrocytes and samples (2 g) were homogenized in 10 ml of isolation-medium 2 containing 0.1 M PEGs of different molecular masses (left panel) or PEG 1500 at different concentrations (right panel). The homogenates were centrifuged at 100,000 g_{max} for 60 minutes. Activities of catalase (■), L-α-hydroxyacid oxidase (◆) and protein content (▲) were determined in the whole homogenate (total activity) and in the supernatant (unsedimentable activity). Columns show the unsedimentable activity of catalase (1) and L-α-hydroxyacid oxidase (2) after homogenization of liver samples in isolation-medium 1. Values are means±s.d. (*n*=3-4). (B) Illustration explaining bottle-stopper experiment. Grey circles, PEGs with different molecular size; black circles, uric acid molecules. (1) PEG molecules that are much smaller than the pore of the membrane channel can therefore freely diffuse with uric acid into peroxisomes; (2) PEG molecules of a size similar to the pore of the channel. PEGs penetrate into the channel very slowly and prevent rapid diffusion of uric acid through the channel; (3) PEG molecules that are too large to penetrate into the channel. Molecules of uric acid easily move through the channel into peroxisomes. (C) Free activity of urate oxidase (■) in purified peroxisomal fractions in the presence of PEGs of different molecular sizes. The incubation medium for determination of urate oxidase activity contained 0.16 M PEGs. Values given are means±s.d. (*n*=3-4). (D) Optical density tracings in swelling experiments with purified peroxisomes incubated at 25°C in the presence of PEGs: (1) control, addition of the buffer only; (2) PEG 200; (3) PEG 400; (4) PEG 600; (5) PEG 1000; (6) PEG 1500. 200 μl of PEG solution (40%, w/v) was added to 600 μl of peroxisomes suspended in isolation-medium 2 (OD_{520}=0.5). The delay in recording, owing to the interruption caused by adding PEGs, was 1 minute. (E) Dependence of OD_{520} value on the molecular size of PEGs. Data were collected 1 minute (■) and 10 minutes (●) after mixing the peroxisomes with PEGs. Results are shown as the difference in OD (ΔOD) of the samples containing PEG relative to the control without solute (zero level). Data of one representative experiment are shown. Experiments with PEGs (C-E) were performed with peroxisomal preparations isolated by the standard procedure.

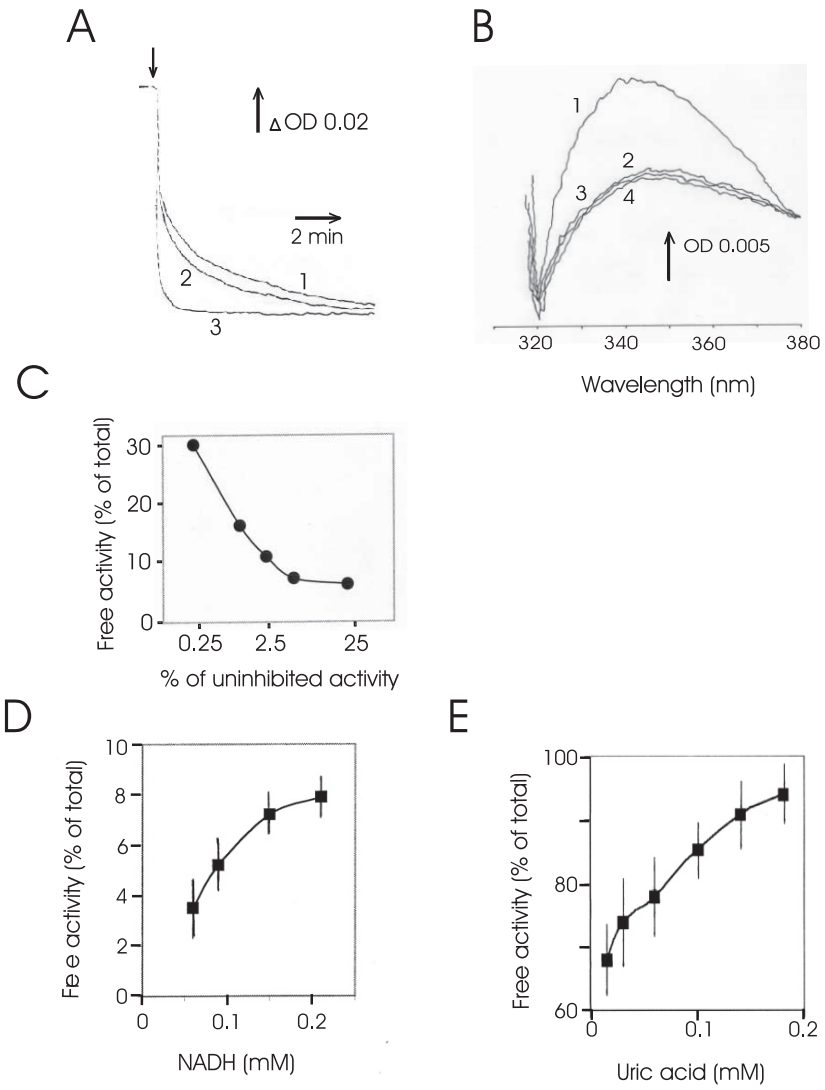

Fig. 4. Cofactors slowly penetrate into peroxisomes. (A) The effect of cofactors on the osmotic behavior of purified peroxisomes. The OD_{520} was recorded at 25°C after mixing a peroxisomal suspension (OD_{520}=0.6) with: (1) NAD^+ or (2) $NADP^+$ (each at 25 mM, final concentration). Control sample (3) was mixed with buffer only (see legend Fig. 3D for further details). (B) Incubation of peroxisomes with cofactors. Suspension of peroxisomes (OD_{520}=0.6) in 20 mM MOPS, pH 7.4 containing 1 mM EDTA was saturated 20 minutes at 25°C with 0.2 mM NADPH (final concentration) in a total volume of 0.8 ml. At the end of incubation, 40 μl of enzyme-substrate mixture (50 mM Tris-Cl, pH 8.0; 0.4 mM 2-oxoglutarate; 0.1 mM ADP; 40 mM ammonium acetate and 10 U/ml glutamate dehydrogenase; each at final concentrations) designed for a sudden oxidation of NADPH was added. The absorbance spectrum (320-380 nm) was measured within 1 minute. The reference sample was incubated in the same conditions as described above, except that the enzyme-substrate mixture was added before the saturation with NADPH. Graphs within B are numbered as follows: (1) sample saturated with NADPH; (2) same as 1 after addition of 5 μl Triton X-100 (10%, w/v); (3) peroxisomes in sample cuvette incubated with NADPH after addition of the enzyme-substrate mixture; (4) same as 3 after addition of Triton X-100. (C) Effect of oxamate on free lactate dehydrogenase activity in a purified peroxisomal fraction. Peroxisomes were treated with 2′,5′-ADP-Sepharose as described (Materials and Methods) and incubated 5 minutes at 25°C with oxamate at different concentrations. Total and free activities of lactate dehydrogenase were immediately determined. Free activity (C-E) was estimated relative to total activity (100%) in the same sample. The measurements were repeated several times. Data from one representative experiment are shown. (D) Dependence of lactate dehydrogenase free activity on NADH concentration. Lactate dehydrogenase activity was measured at different NADH concentrations and a fixed concentration of pyruvate (1.0 mM, final concentration). Values given are means±s.d. (n=4-5). (E) Dependence of urate oxidase free activity on uric acid concentration. Urate oxidase forms a crystalline structure inside peroxisomes called a nucleoid (Masters and Crane, 1995); this explains the absence of leakage of the enzyme from broken particles. Presumably almost all the free activity of urate oxidase arises from the enzyme that is present inside the particles.

that the free activity of lactate dehydrogenase increases gradually with an increased NADH content in the sample. This indicates a dependence of the enzyme activity inside peroxisomes on the concentration of cofactor outside the particles (Fig. 4D). As a whole, the results imply that, in vitro the cofactor-dependent enzymes in peroxisomes are not saturated by cofactors, owing to a restriction in the permeability of the peroxisomal membrane to these compounds. Interestingly, a similar correlation was also detected when the total and the free activities of urate oxidase were measured at low uric acid concentrations (Fig. 4E).

The selection of solutes based on their different rates of permeation through the membrane may determine the functional behavior of peroxisomes in vivo (see Discussion for further details). Therefore, it was important to assess the difference between the rates of diffusion into peroxisomes for cofactors and for common metabolites. The latencies of peroxisomal lactate dehydrogenase and urate oxidase (see Fig. 4D,E) indicate that these enzymes may not be saturated inside peroxisomes, which implies dependence of their activities on substrate (cofactor) concentrations in the particles. Consequently, the level of free enzyme activity might reflect the substrate (cofactor) concentration inside peroxisomes. Preliminary assessments show that the difference in the rate of permeation through the peroxisomal membrane for uric acid (168 Da) and NADH (663.4 Da) may reach a level up to 200-fold. Attempts to verify these estimations by using swelling experiments faced difficulties because of the high membrane permeation rate of small solutes was beyond the detection ability of conventional technique. For this reason we used a stopped-flow apparatus to register peroxisomal swelling in the presence of a compound with relatively low molecular mass (PEG 200) within 20 seconds. Although the obtained results are only semi-quantitative (data not shown) they indicate that the difference between solutes of a size comparable to PEG 200 and molecules such as cofactors (NAD^+ or $NADP^+$) in the time to reach an

equilibrium of their concentrations inside and outside peroxisomes might be more than 100-fold.

Discussion

The data presented here allow two major conclusions. (1) The main function of the mammalian peroxisomal membrane as a permeability barrier is to provide effective separation of bulky cofactors (NAD/H, NAD(P)/H and CoA) from smaller metabolic intermediates. (2) The difference in molecular size between cofactors and common metabolites plays an important functional role and offers a simple method to compartmentalize these compounds by biological membranes. As a whole, our results reconcile the opposing views on the barrier function of peroxisomal membrane, which has been a matter of controversy for more than four decades (see Introduction). Moreover, it is now obvious that the bulk of published experimental results related to this field is not as contradictory as so far thought. For instance, the conflicting results concerning the latency of certain peroxisomal enzymes (Antonenkov, 1989; Wolvetang et al., 1990; Verleur and Wanders, 1993) can be easily explained by the observed link between this phenomenon and the dependence of these enzymes on cofactors.

Our data confirm the size-exclusion limit for permeability of peroxisomal membrane (about 1000 Da) that was determined previously (see Introduction). More importantly, the results revealed a large difference in the rate of permeation between cofactors and common metabolites. The membrane barrier does not restrict diffusion (at least to an extent of physiological importance) of small water-soluble metabolites, such as substrates and products of different peroxisomal enzymatic reactions. These data suggest that in vivo, peroxisomes share a common pool for small solutes with the surrounding cytoplasm. However, the rate of permeation for cofactors is limited. It may be high enough to avoid the requirement of more costly mechanisms to deliver cofactor molecules, such as specific transmembrane transporters, into peroxisomes. However, the slow rate of exchange between cofactors located outside and inside peroxisomes points against a direct involvement of this process in one of the basic functions of the peroxisomal metabolic system: the export and import of redox equivalents into and out of peroxisomes, and also the transport of acyl-/acetyl-groups across the membrane. Instead, the permeability properties of the peroxisomal membrane require that these processes occur via shuttle systems (see below). One can expect that these systems ensure a unique functional state of cofactors inside peroxisomes such as the relative levels of reduced and oxidized forms of NAD or NADP, as well as the relative proportions of free CoA and its acyl-derivatives. Therefore it is reasonable to assume the presence of a separate pool of cofactors in peroxisomes that is functionally independent from the corresponding pool of cofactors in the surrounding cytoplasm.

It is generally accepted that the main metabolic pathways in mammalian peroxisomes lead to: (a) the reduction of NAD^+; (b) the oxidation of NADPH and (c) the formation of acyl-/acetyl-CoAs (reviewed in Masters and Crane, 1995; Mannaerts et al., 2000; Wanders et al., 2001; Hiltunen et al., 2003). The resulting compounds (NADH, $NADP^+$ and acyl-CoAs) have to be reconverted to avoid their accumulation in peroxisomes because the direct export of these molecules out of peroxisomes by means of diffusion through the membrane barrier seems very slow (see above). Increasing evidence indicates that the metabolic conversion of peroxisomal cofactors proceeds via shuttle mechanisms that construct export/import systems across the peroxisomal membrane (Antonenkov, 1989; Van Roemund et al., 1995; Van Roemund et al., 1998; Van Roemund et al., 1999; Baumgart et al., 1996; Henke et al., 1998; Kal et al., 1999). Several NAD- (glycerol 3-phosphate dehydrogenase, lactate dehydrogenase) and NADP- (isocitrate dehydrogenase, glucose 6-phosphate dehydrogenase) dependent dehydrogenases as well as carnitine-acyltransferases that have been found in peroxisomes are possible participants of the shuttle systems. Importantly, the substrates for all of these enzymes are small water-soluble molecules that can freely penetrate the peroxisomal membrane.

Our data provide an unexpected view on the functional role of peroxisomal nudix hydrolases. The major substrates for this family of enzymes are nucleoside diphosphates linked to some other moiety, x, hence the acronym 'Nudix' (Bessman et al., 1996). At least two members of this family are located in peroxisomes (Cartwright et al., 2000; Abdelraheim et al., 2001); one is active towards CoA and some of its derivatives, whereas the other one hydrolyzes NADPH. Interestingly, both hydrolases cleave cofactor molecules into two parts of approximately equal size. Therefore, the nudix hydrolases transform one bulky cofactor molecule into two smaller molecules of sizes similar to common metabolites, providing a route for the removal of cofactors out of peroxisomes.

It might be difficult to reconcile the presence of solute transporters side by side with large, nonselective channels in the same peroxisomal membrane. One explanation to this apparent contradiction might be that the transporter carries solutes of comparable size with cofactors. The only experimentally demonstrated example of peroxisomal solute transporters is Ant1p, an ATP/AMP antiporter from the yeast *Saccharomyces cerevisiae* (Palmieri et al., 2001) and its mammalian counterpart PMP 34 (Visser et al., 2002). The molecular mass of ATP (507.2 Da) is large enough to predict a restriction in diffusion of this compound through the peroxisomal membrane. Acceleration in the transfer of nucleotides by means of an antiporter (ATP into peroxisomes, AMP out of the particles) may provide a shift in the steady-state ratio of ATP-AMP inside peroxisomes in favor of ATP, owing to limitations in its free diffusion out of the peroxisome.

The precise molecular mechanism of peroxisomal membrane permeation is not clear. The most attractive explanation for the above data is the presence of at least two types of channels in the peroxisomal membrane. One of them, with a size-exclusion-limit of about 1000 Da, allows the slow diffusion of cofactors as well as the relatively fast penetration of common metabolites. The other channel is too small for cofactors but large enough for common metabolites. The ratio of these two channels in the membrane might favor of a much higher rate of permeation of common metabolites than cofactors. The channels provide an easy passage for common metabolites through the peroxisomal membrane, whereas at least some bulky organic molecules are in need of specific transmembrane transporters.

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