The behavior of peroxisomes in vitro: mammalian peroxisomes are osmotically sensitive particles

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Antonenkov, Vasily D., Raija T. Sormunen, and J. Kalervo Hiltunen. The behavior of peroxisomes in vitro: mammalian peroxisomes are osmotically sensitive particles. Am J Physiol Cell Physiol 287: C1623–C1635, 2004. First published August 11, 2004; doi: 10.1152/ajpcell.00142.2004.—It has been known for a long time that mammalian peroxisomes are extremely fragile in vitro. Changes in the morphological appearance and leakage of proteins from purified particles demonstrate that peroxisomes are damaged during isolation. However, some properties of purified peroxisomes, e.g., the latency of catalase, imply that their membranes are not disrupted. In the current study, we tried to ascertain the mechanism of this unusual behavior of peroxisomes in vitro. Biochemical and morphological examination of isolated peroxisomes subjected to sonication or to freezing and thawing showed that the membrane of the particles seals after disruption, restoring permeability properties. Transient damage of the membrane leads to the formation of peroxisomal “ghosts” containing nucleoid but nearly devoid of matrix proteins. The rate of leakage of matrix proteins from broken particles depended inversely on their molecular size. The effect of polyethylene glycols on peroxisomal integrity indicated that these particles are osmotically sensitive. Peroxisomes suffered an osmotic lysis during isolation that was resistant to commonly used low-molecular-mass osmoprotectors, e.g., sucrose. Damage to peroxisomes was partially prevented by applying more “bulky” osmoprotectors, e.g., polyethylene glycol 1500. A method was developed for the isolation of highly purified and nearly intact peroxisomes from rat liver by using polyethylene glycol 1500 as an osmoprotector.

Peroxisomes are essential organelles present in virtually all eukaryotic cells. These spheroid particles contain matrix consisting of soluble proteins, surrounded by a single membrane. Contrary to their morphological simplicity, peroxisomes are metabolically complex because of their diverse enzyme content, which depends on species, tissues, nutritional conditions, and developmental stage (20, 25, 26, 34). The behavior of peroxisomes under in vitro conditions does not follow the rules applicable to other cellular organelles. In gradients formed from Percoll (colloidal suspension of silica), peroxisomes exhibit a density that is lower or comparable with the density of mitochondria and lysosomes. In contrast, peroxisomes possess a higher equilibrium density than other organelles in gradients formed from low-molecular-mass media such as sucrose, Nycodenz, or iodixanol, indicating that these compounds may penetrate the peroxisomal membrane (9, 17, 26, 35, 38). Several enzymes confined to peroxisomes show no latency (catalase is an exception, see Ref. 29), suggesting that the boundary membrane of these organelles is freely permeable to small solute molecules (8, 12, 36, 37). This suggestion was supported by experiments showing little, if any, effect of iso-osmotic sucrose solutions on the stability of peroxisomes during isolation (8, 12). These observations have led to the conclusion that mammalian peroxisomes do not behave as osmotically sensitive particles in low-molecular-mass solutes (8, 12, 17, 26, 29).

The fragility of peroxisomes under in vitro conditions is well known, but the mechanism of this phenomenon is poorly understood. Soluble peroxisomal matrix proteins are readily released during the isolation of peroxisomes, and the resulting purified fraction appears less electron dense than the organelles in situ (2, 9, 11, 17, 21, 23, 35, 38, 40). On the other hand, a large number of observations show latency of catalase in the purified peroxisomal fraction, and this is considered to confirm that the isolated particles are intact (see, for example, Refs. 27 and 38). The uncertainty surrounding the quality of isolated peroxisomes diminishes the validity of in vitro experiments with these particles. In addition, disruption of the particles and leakage of their constituents decreases the efficacy of newly developed approaches, like proteomics, to study the content of mammalian peroxisomes (21).

Our recent studies on the permeability properties of the rat liver peroxisomal membrane indicate that it allows free access into the particles to small, water-soluble metabolites, e.g., substrates for peroxisomal enzymes (urate, glycolate), while restricting penetration of more “bulky” organic molecules, such as cofactors [NAD(P)H, CoA; Antonenkov VD, unpublished observations]. This feature of the membrane may determine the behavior of peroxisomes as true osmometers (see Discussion for more details). Osmosis may cause damage to the particles during their isolation that can, at least partially, explain the in vitro fragility of mammalian peroxisomes.

Here we describe experiments aimed at revealing the mechanisms of the “bizarre” behavior of mammalian peroxisomes in vitro. Our data indicate that peroxisomes, like other cellular organelles, are osmotically sensitive particles and may undergo osmotic lysis during isolation. The lysis is accompanied by a leakage of soluble matrix proteins through the broken membrane. The level of this leakage is inversely proportional to the size of the molecules. The rupture of the peroxisomal membrane is transient and reversible, resulting in the formation of peroxisomal “ghosts.” A new method based on exploiting polyethylene glycol 1500 (PEG 1500) as an osmoprotector was used to stabilize peroxisomes from osmotic lysis, allowing in vitro isolation of highly purified and intact peroxisomes from rat liver.

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developed. It allowed for the isolation of highly purified rat liver peroxisomes that were filled with soluble matrix proteins.

MATERIALS AND METHODS

Materials. Optiprep density gradient medium, a 60% (wt/vol) solution of iodixanol in water, Nycodenz, sucrose, PEGs, proteinase K, BSA, phenylmethylsulfonyl fluoride (PMSF), and alkaline phosphatase conjugated anti-rabbit IgG were obtained from Sigma (St. Louis, MO). Chemicals for the detection of immunoblots of alkaline phosphatase conjugated secondary antibodies were from Boehringer (Mannheim, Germany). Fixatives and other reagents for electron microscopy were from Electron Microscopy Sciences (Fort Washington, PA). Other chemicals used were of proanalysis grade.

Animals and purification of peroxisomes. The use of experimental animals was approved by the committee on animal experimentation at the University of Oulu. Male Sprague-Dawley rats weighing 200–250 g were fasted overnight. Animals were killed with an overdose of carbon dioxide. The livers were perfused via the portal vein. For the purification of peroxisomes by the “conventional” procedure, the livers were homogenized in isolation medium-1 [1:4, wt/vol; 0.25 M sucrose, 10 mM MOPS, pH 7.4, 1 mM EDTA, 1 mM EGTA, 2 mM dithiothreitol (DTT), 0.1% (vol/vol) ethanol, and 0.1 mM PMSF]. The homogenates were subjected to differential centrifugation followed by centrifugation in a self-generating Percoll gradient (see Ref. 6 for further details). Fractions enriched in peroxisomes (from the top of the gradient) were collected (total volume 16 ml) and loaded on a multistep Nycodenz gradient [two tubes, each containing 32 ml of gradient) were collected (total volume 16 ml) and loaded on a centrifugation in a vertical rotor VTi50 (Beckman, Palo Alto, CA) at 100,000 g max for 40 min in a vertical rotor (type VTi65; Beckman). The gradient solutions contained 10 mM MOPS, pH 7.4, 1 mM EDTA, and 0.1% (vol/vol) ethanol. To remove the isolation medium containing protease inhibitors, peroxisomes were sedimented, and the pellet was suspended in 20 mM MOPS, pH 7.4. The aliquots (0.4 mg protein/ml) were incubated with 0.6 U/ml proteinase K at 37°C. The reaction was quenched with 1 mM PMSF.

Purified rat liver peroxisomes show no detectable alcohol dehydrogenase activity with ethanol as a substrate (Antonenkov, unpublished observation). Therefore, we used alcohol dehydrogenase from baker’s yeast (homotetramer, molecular mass 140 kDa; Sigma) to assess the accessibility of broken peroxisomes to external proteins. A suspension of purified peroxisomes (0.4 mg protein/ml) mixed with alcohol dehydrogenase (0.01 mg/ml, final concentration) was sonicated (2 × 15 s) and, after the addition of KCl to a final concentration 0.15 M (to prevent nonspecific “adhesion” of soluble proteins on the surface of the peroxisomal membrane), was centrifuged in a multistep sucrose gradient as described above. Gradient fractions enriched with peroxisomal ghosts containing alcohol dehydrogenase were subjected to a second sucrose gradient centrifugation. The final preparation of peroxisomal ghosts was diluted by the dropwise addition of isolation medium-2, and the particles were sedimented. The pellet was resuspended in isolation medium-2 or in 20 mM MOPS, pH 7.4 (for treatment by proteinase K). Control peroxisomal samples were treated as described, except for sonication.

Assay of enzymes and latency determination. Marker enzyme activities were measured by using standard procedures (3, 6, 9, 23) to determine the localization in the gradient fractions of mitochondria (glutamate dehydrogenase), lysosomes (acid phosphatase), endoplasmic reticulum (esterase), cytosolic proteins (phosphoglucose isomerase), peroxisomes, and their following constituents: matrix proteins (catalase, l-α-hydroxycid acid oxidase), membrane (NADH-cytochrome c reductase), and nucleoid (urate oxidase). The high ultraviolet absorbance of iodixanol interfered with measuring catalase and urate oxidae activities by standard methods (UV absorbance determination) in the fractions containing Optiprep. Therefore, for the detection of these enzymes in the Optiprep gradients, we used colorimetric procedures (9, 35). The activity of alcohol dehydrogenase was monitored by the reduction of NAD+ by ethanol. Lactate dehydrogenase activity was measured in the presence of pyruvate and NADH (10). The thiolic cleavage activity of peroxisomal fractions toward aceatoctyl-CoA was determined at 306 nm (ε: 3,600 M−1/cm). In normal rat liver peroxisomes, the bulk of this activity is determined by soluble matrix 3-oxoacyl-CoA thiolase (4, 6). Units of enzyme activity are expressed as micromoles of substrate consumed or product formed per minute. Catalase units were defined as described (23).

The latency of the enzymes in the peroxisomal fraction was determined by comparing “free” and “total” activities. The free activity was measured at 25°C in the standard assay mixture for the corresponding enzyme. After recording this activity (2–5 min), the membrane barrier was disrupted using Triton X-100 (0.05% wt/vol, final concentration), and the total enzyme activity was detected. To prevent the interference of enzymes escaping from broken particles on latency determination, in some experiments the freshly isolated peroxisomes were sedimented in the presence of 0.15 M KCl, and the pellet was suspended in isolation medium-2. The “unseeddable” activity of the enzymes was measured in supernatants obtained after centrifugation of peroxisomes at 100,000 g max for 60 min. Protein was determined with BSA as standard (32).

Electron microscopy. Isolated peroxisomes were fixed with 1% (wt/vol) glutaraldehyde (final concentration), as described in the legends to Figs. 1 and 7. After fixation, the organelles were pelleted by centrifugation at 20,000 g max for 30 min. The pellets were post-
fixed with 1% (wt/vol) OsO4 in 0.1 M PIPES (pH 7.4) for 1 h and stained with 1% (wt/vol) uranyl acetate for 1 h. The samples were then 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.

Other methods. Proteins were separated by SDS-PAGE under reducing conditions, with 15% (wt/vol) acrylamide gels (Criterion Precast Gel; Bio-Rad, Hercules, CA). Silver staining of the gels was performed as described (28). For immunoblot analysis, the proteins separated by SDS-PAGE were transferred to nitrocellulose (0.45 μm Protran; Schlechter & Schuell, Dassel, Germany), and the blot was incubated with the primary antibodies, followed by detection with alkaline phosphatase-labeled anti-rabbit IgG (28). Polyclonal antibodies were raised in rabbits against bovine liver catalase and glutamate dehydrogenase (purchased from Chemicon, Temecula, CA), alcohol dehydrogenase from baker’s yeast and human glutathione 3-transferase A 1–1 (Calbiochem, La Jolla, CA), rat peroxisomal 3-oxoacyl-CoA thiolase (thiolase; see Refs. 6 and 7), rat peroxisomal 3-oxoacyl-CoA thiolase (our own observation, also see Ref. 38). Purified peroxisomes contain a matrix of molecular mass 22 kDa (PMP 22; NH2-APAASRLRVESELG), and the COOH-terminal sequence of rat liver inner mitochondrial membrane enzyme α-3-hydroxybutyrate dehydrogenase (THFPGAISDKIYIH-COOH).

RESULTS

Broken peroxisomal membrane reseals. In initial experiments, we used a peroxisomal fraction isolated by means of Nycodenz gradient centrifugation (the conventional technique). The purity of peroxisomes was estimated at 90–95% from the specific activities of the marker enzymes, as described (15). In agreement with these data, an electron microscopic examination revealed a nearly homogeneous population of peroxisomes (Fig. 1A).

As previously discussed, the isolation of mammalian peroxisomes is accompanied by a leakage of soluble matrix proteins, indicating partial destruction of the particles (2, 9, 21, 23, 26, 27, 35, 38). Purified peroxisomes contain a matrix of variable electron density (2, 9, 23, 27, 35, 38; also see Fig. 1, A, B, and F), confirming damage of the organelles. However, in spite of this, preparations of purified particles usually show a high level of catalase latency (8, 12, 27, 38).

To find an explanation for these contradictory observations, we exposed purified peroxisomes to sonication and determined
the free and unsedimentable activities of catalase and cofactor-dependent enzymes to detect the extent of peroxisomal disruption (Fig. 2, Aa–Ac). As expected, the free activity of the enzymes tested gradually increased in the peroxisomal preparation upon sonication, accompanied by an elevation in the unsedimentable activity. The gradual leakage of soluble matrix proteins from peroxisomes was confirmed by immunoblotting experiments (Fig. 2Ad). However, when we measured the free activity of enzymes in the sediment fractions obtained by centrifugation of the broken particles, it was persistently low, independent of the intensity of sonication (see Fig. 2, Aa–Ac, values marked by triangles).

The data suggest that the free activity detected in purified peroxisomal preparations is determined mainly by the enzymes escaping from broken particles and does not reflect an increase in the permeability of peroxisomal membrane to substrates and/or cofactors. To confirm this supposition, we used peroxisomes damaged by freezing and thawing and incubated them with proteinase K. As expected, treatment by two cycles of freezing and thawing leads to an increase in the free activity of catalase (data not shown) and lactate dehydrogenase (Fig. 2B), which reflects destruction of the particles. Incubation of this particle preparation with protease diminished the free activity to the near-zero level while only partially affecting the total activity of the enzymes. The activity resistant to proteinase K resides inside peroxisomes, since it was recovered in the sediment by recentrifugation of the organelles (data not shown).

The results obtained indicate that the free activity is determined by enzymes that have leaked out of the particles. This conclusion implies damage of the peroxisomal membrane. In contrast, the latency of the enzymes resistant to protease treatment (i.e., which are present inside the particles) indicates intactness of the membrane. To explain these contradictory observations, two main proposals may be considered: 1) damage of peroxisomes resulting from different treatments leads to total destruction of the particles; in this case, one can expect the simultaneous presence of intact peroxisomes and remnants of the broken particles in the treated samples; 2) moderate damage of peroxisomes results in formation of temporal holes or splits in the membrane that allow partial leakage of matrix proteins; when the destructive treatment is over the broken membrane reseals and the latency of the enzymes remaining inside the particles is restored. Irreversible damage of peroxisomes (first model) may lead to formation of a complex mixture composed of intact particles, membrane fragments, free nucleoids, and soluble matrix proteins. However, after resealing of the membrane (second model), one can expect the appearance of peroxisomal ghosts containing membrane, nucleoid, and some part of matrix proteins.

Recentrifugation of freshly purified peroxisomes in a sucrose gradient showed comigration of marker enzymes for peroxisomal matrix (catalase), nucleoid (urate oxidase), and membrane (NADH-cytochrome c reductase) in the region of gradient at a density of 1.21–1.23 g/cm³, indicating integrity of the particles (Fig. 3A). Moderate damage of peroxisomes (2 cycles of sonication) was accompanied by partial release of the matrix enzymes [catalase (Fig. 3B) and lactate dehydrogenase (data not shown)] that were retained on top of the gradient. The remainder of these enzymes (40–50% of the total content in the gradient) was associated with particles with a lower equilibrium density (1.20–1.21 g/cm³) than untreated peroxisomes. Importantly, matrix enzymes that remained in the particles were resistant to proteinase K treatment (data not shown) and showed latency (see legend to Fig. 3B). Vigorous sonication of peroxisomes (6 cycles) resulted in their complete destruction. Gradient centrifugation of...
the broken particles indicated clear separation of matrix proteins, membrane fragments, and nucleoids (Fig. 3C).

Morphological examination of the moderately sonicated peroxisomes (2 cycles; Fig. 1, C and G) revealed the presence of peroxisome-like particles (peroxisomal ghosts) containing nucleoids and apparently intact membrane. However, the electron-dense material in the matrix was less visible than in peroxisomes without sonication (see Fig. 1B for comparison). More vigorous sonication of peroxisomes (4 cycles; Fig. 1D) was accompanied by the appearance of free nucleoids extruded from the particles and small, sealed membrane structures. Further sonication (6 cycles) led to the formation of tiny membrane bubbles (Fig. 1, E and H).

The data indicate that moderate mechanical damage of peroxisomal membrane may be a reversible process that is accompanied by leakage of some matrix proteins and by formation of peroxisomal ghosts. To provide further evidence supporting this model, we introduced an artificial enzyme (bacterial alcohol dehydrogenase) into the ghosts by sonicating a mixture of purified organelles with alcohol dehydrogenase. These experiments show that a portion of the “artificial” enzyme activity comigrated with the peroxisomal ghosts in sucrose gradients (Fig. 4A). This activity was latent (see legend to Fig. 4A) and resistant to proteolytic degradation (Fig. 4, B and C).

Damage of peroxisomes during isolation can be prevented by using an appropriate osmoprotector. Our data describing the permeability properties of peroxisomal membrane (unpublished observations) indicate that the membrane allows easy
access into the particles to compounds with molecular size <300–400 Da, whereas the rate of permeation for larger molecules is heavily restricted. This feature of peroxisomes implies that the particles may be exposed to osmotic pressure resulting from differences in the rate of diffusion through the membrane between water (high flow rate into the particles) and some metabolites (slow diffusion out of peroxisomes). The osmotic imbalance may cause damage of peroxisomes during tissue homogenization and subsequent fractionation (see DISCUSSION for further details). Importantly, the molecular sizes of sucrose (molecular mass 342 Da) and other commonly used osmoprotectors is small enough to allow their easy diffusion

Fig. 5. Effect of polyethylene glycol (PEG) on the integrity of peroxisomes in vitro. A: effect of PEG on solubilization of enzymes from peroxisomes during rat liver homogenization. Liver samples (2 g) were homogenized in 10 ml isolation medium-2 containing 12% (wt/vol) PEG with different molecular masses (left) or PEG 1500 at different concentrations (right). The homogenates were centrifuged at 100,000 g, for 60 min. Activities of catalase (●), 1-α-hydroxyacid oxidase (■), phosphoglucone isomerase (○, cytoplasmic marker), and protein content (△) were measured in the whole homogenate (total activity) and in the supernatants (unsedimentable activity). The unsedimentable activity is presented as a percentage of the control (100%) without PEG. B and C: effect of PEG 1500 on subcellular distribution of peroxisomal proteins. Rat liver homogenates were prepared in the presence of 0.25 M sucrose (Ba and Ca–Cc, top) or 12% (wt/vol) PEG 1500 (Bb and Ca–Cc, bottom) and fractionated by differential centrifugation into a nuclear (N), a heavy mitochondrial (M), a light mitochondrial (L), a microsomal (P), and a cytosolic (S) fraction. The distribution of peroxisomal proteins was determined by urate oxidase (a) and catalase (b) activity (B) or by immunoblot analysis (C) with antibodies raised against SCP-2 (a), thiolase (b), and 22-kDa peroxisomal integral membrane protein (PMP 22; c). Relative specific activities of the enzymes tested are presented vs. cumulative percentage of total protein (B). Overall recoveries for the enzymes were between 98 and 114%. Immunoblotting (C) was performed with an equal quantity of protein from each fraction loaded on a gel (2–15 μg, depending on the specific antibody used). Note the enrichment of subcellular fractions containing peroxisomes (N, M, and L) in soluble matrix proteins (catalase, thiolase, and SCP-2) resulting from application of PEG 1500 instead of sucrose as a potential osmoprotector. D: catalase activity distribution in Percoll gradients after centrifugation of the “light” mitochondrial fractions isolated in the presence of 0.25 M sucrose (gray bars, the enzyme recovery was 92%) or 12% (wt/vol) PEG 1500 (filled bars, 108%). A suspension of the light mitochondrial fraction (80 ml) was loaded on a layer of 50% (wt/vol) Percoll (100 ml) containing 12% (wt/vol) PEG 1500 and 0.25 M sucrose, and the density gradient was generated by centrifugation at 23,500 g for 60 min. Note the increase in equilibrium density of peroxisomes as a result of their isolation in the presence of PEG 1500. E: catalase distribution after Nycodenz density gradient centrifugation of the “postnuclear” rat liver homogenate (dilution 1:4, wt/vol) prepared on isolation medium-2 containing 0.25 M sucrose (gray bars) or 12% (wt/vol) PEG 1500 (filled bars). The homogenates were centrifuged at 1,800 g, for 12 min to remove nuclear and cell debris, and the resulting supernatants were loaded on multistep Nycodenz gradients. The gradients were centrifuged at 100,000 g, for 90 min. The activities of catalase (E) and 1-α-hydroxyacid oxidase (data not shown) were determined as markers for soluble matrix proteins. Analysis of the distribution of nucleoids (urate oxidase activity determination) and peroxisomal membrane (immunodetection of PMP 22) revealed the presence of these peroxisomal constituents near the bottom of gradients, indicating that only the soluble matrix proteins leak out of peroxisomes during homogenization (data not shown). The recoveries of the enzymes were 92–116%.
through the membrane, diminishing the osmoprotective effect of these compounds relative to peroxisomes. To confirm this assumption, a comparative study of peroxisomal integrity under in vitro conditions using media containing 0.25 M sucrose or PEG 1500 was undertaken. We expected that PEG 1500, which showed a very low rate of transmembrane diffusion (Antonenkov VD, unpublished observation), may decrease the osmotic damage of peroxisomes, preventing an abrupt access of water in the particles during tissue homogenization.

As shown in Fig. 5A, the rate of leakage of soluble matrix proteins from broken peroxisomes as a result of tissue homogenization depends on the presence of PEGs in the isolation medium. Leakage gradually decreased with an increase in the molecular mass of PEGs used or with an increased concentration of PEG 1500. The protective effect of PEG 1500 on the integrity of peroxisomes was confirmed after differential centrifugation of rat liver homogenate (Fig. 5, B and C). In control samples (homogenates prepared on 0.25 M sucrose), the bulk of the soluble matrix proteins escaped from peroxisomes and was detected in a cytosolic fraction, in agreement with the data reported by others (9, 17, 23, 26, 35, 38). In the presence of PEG 1500, the level of matrix proteins in the cytosolic fraction was reduced significantly. Further confirmation of the preserving effect of PEG 1500 on peroxisomes was obtained by means of Percoll gradient centrifugation (Fig. 5D). The “light” mitochondrial fraction enriched in peroxisomes was used to assess an equilibrium density of the particles. Peroxisomes from the control samples did not penetrate in the Percoll gradient and had an equilibrium density of 1.040–1.050 g/cm³, corresponding to data described previously (6, 17). In contrast, peroxisomes isolated in the presence of PEG 1500 were concentrated mainly near the bottom of the gradient (equilibrium density 1.060–1.090 g/cm³), implying that the particles are more densely packed with matrix proteins than control samples.
Finally, rat liver postnuclear homogenate was centrifuged in a multistep Nycodenz gradient to directly show the relative distribution between peroxisomes and cytoplasm of the soluble matrix enzymes catalase (Fig. 5E) and l-α-hydroxyacid oxidase (data not shown). The results indicate a high fragility of peroxisomes during tissue homogenization in the presence of 0.25 M sucrose, whereas PEG 1500 effectively prevented damage of the particles.

Separation of intact and broken peroxisomes by means of Percoll gradient centrifugation. The data described above led to the conclusion that sucrose and other commonly used osmoprotectors are ineffective at preventing damage to peroxisomes during isolation. In our preliminary experiments, we found that, although PEG 1500 is an appropriate protector relative to peroxisomes, it failed to completely preserve other subcellular particles (mitochondria, lysosomes). Therefore, we searched for an optimal isolation medium suitable for different organelles. The best results were obtained by using a mixture of 12% (wt/vol) PEG 1500 and 0.16 M sucrose. To separate intact peroxisomes from peroxisomal ghosts, we exploited the difference in their equilibrium density in Percoll gradients. After homogenization of the perfused rat liver, the nuclei and cell debris were removed by low-speed centrifugation, and the “postnuclear” homogenate was directly loaded on the Percoll solution. Figure 6 shows that, after centrifugation in Percoll containing PEG 1500 and sucrose, the peroxisomes were concentrated near the bottom of the self-generated gradient, whereas the other organelles were found mainly in the top fractions. According to enzymatic (Table 1) and morphological (Fig. 7, A and B) analysis, the sample enriched in peroxisomes still contains a substantial amount of lysosomes and mitochondria, while being poor in fragments of endoplasmic reticulum (microsomes). The enrichment in peroxisomes is ~10-fold over the original homogenate (Table 1). Morphological examination indicates that the peroxisomes in the bottom gradient fractions appear as intact particles filled with matrix proteins (Fig. 7A), whereas in the top fractions they resemble mostly the peroxisomal ghosts (Fig. 7B).

Isolation of highly purified intact peroxisomes. To further separate peroxisomes from other cellular organelles, the sample from the bottom of a Percoll gradient enriched with intact peroxisomes was subjected to Optiprep gradient centrifugation. Marker enzymes activity determination (Fig. 8A), immunoblot analysis (Fig. 8B), and silver staining of proteins separated by SDS-PAGE (Fig. 8C) showed that peroxisomes are located close to the center of the gradient (fractions 4–7) in a density range 1.18–1.21 g/cm³ and well separated from the other organelles, mainly mitochondria and lysosomes, which are responsible for the major protein content in the sample (gradient fractions 13–15). Only a small part of the peroxisomal

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Table 1. Isolation of intact peroxisomes

<table>
<thead>
<tr>
<th>Enzyme (protein)</th>
<th>Absolute Activity in Homogenate, U/g liver</th>
<th>Composition of Fractions, % Activity in Homogenate</th>
<th>Relative Specific Activity in Purified Peroxisomal Fraction With Respect to Whole Homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, mg/g liver</td>
<td>202.4±16.2</td>
<td>2.8±0.6</td>
<td>0.28±0.07</td>
</tr>
<tr>
<td>Catalase</td>
<td>51.2±4.6</td>
<td>28.7±4.2</td>
<td>11.0±1.2</td>
</tr>
<tr>
<td>Urate oxidase</td>
<td>3.6±0.9</td>
<td>32.8±6.3</td>
<td>11.6±0.9</td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>10.4±2.5</td>
<td>4.7±1.4</td>
<td>0.009±0.004</td>
</tr>
<tr>
<td>Esterase</td>
<td>366.0±28.5</td>
<td>0.6±0.2</td>
<td>0.007±0.002</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>225.4±25.2</td>
<td>7.6±1.2</td>
<td>0.012±0.004</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 3–4 experiments. Enzyme activity in purified peroxisomal fraction was measured before resedimentation of the particles (see MATERIALS AND METHODS).
matrix enzyme activity was found in the upper layer of the gradient (fractions 13–15), indicating that damage to the particles during centrifugation was negligible. This was confirmed by electron microscopic examination of the gradient fractions enriched in peroxisomes (Fig. 7C). Most particles show a dense granular matrix surrounded by a distinct membrane. Estimated by peroxisomal marker enzymes, the organelles were purified 38– to 40-fold over the original homogenate (Table 1). On a

Fig. 8. Optiprep gradient centrifugation of subcellular organelles. Fractions from the bottom of a Percoll gradient enriched with intact peroxisomes were loaded on an Optiprep multistep gradient and centrifuged as described under MATERIALS AND METHODS. A: fractions were analyzed for the following marker enzyme activities: catalase (a, filled bars), L-α-hydroxyacid oxidase (a, gray bars), urate oxidase (a, open bars), glutamate dehydrogenase (b, filled bars), acid phosphatase (b, gray bars), esterase (b, open bars), or protein content (c). B and C: proteins from equal volumes of each fraction were separated by SDS-PAGE and immunoblotted (B) or silver stained (C). The relative molecular mass (Mr) of marker proteins is indicated on left in C. The asterisk in C indicates the position in the gel of urate oxidase monomer (34 kDa). The molecular masses of native peroxisomal proteins that were used to compare the rate of their leakage from the broken particles are as follows: catalase, tetramer of 240 kDa (26); lactate dehydrogenase, tetramer of 140 kDa (10); SCP-2/thiolase, two dimeric isoforms of 116 and 104 kDa (5); L-α-hydroxyacid oxidase, dimer of ~100 kDa (22); thiolase, dimer of 80 kDa (7); SCP-2, monomer of 12.5 kDa (16). Monomers of urate oxidase form a large crystalline structure in peroxisomes (nucleoids; see Refs. 2 and 26). PMP 22 is an integral membrane protein that is present in peroxisomes only (18).
protein basis (15), peroxisomes contribute >95% of the total protein content of the fraction.

The size of proteins determines the rate of their leakage from peroxisomes. Most peroxisomal matrix enzymes easily escape from broken particles, indicating that they are present inside the organelle in a soluble form. However, matrix proteins may leak out from peroxisomes with different rates (2, 16, 26, 33). To understand this process, we undertook a more detailed investigation of this phenomenon.

Analysis of peroxisomal proteins throughout the Optiprep gradient revealed heterogeneity in their distribution (Fig. 8). For example, immunodetection of SCP-2 visualizes only a band in fractions 4–7 containing “heavy” peroxisomes (Fig. 8B). In contrast, the activities of catalase and urate oxidase (Fig. 8A), the immunosignal to PMP 22 (Fig. 8B), and the protein band reflecting the position of peroxisomal nucleoids (see Fig. 8C) were broadly distributed throughout the whole gradient. Localization of SCP-2 is clearly different compared with that of SCP-2/3-oxoacyl-CoA thiolase (SCP-2/thiolase), an enzyme that contains the full sequence of SCP-2 as a COOH-terminal part and that can be detected on the same immunoblot by the antibodies generated against SCP-2 (31).

The phenomenon of heterogeneous distribution of peroxisomal proteins throughout gradients formed by different media (sucrose, Nycodenz, Optiprep) is usually attributed to heterogeneity of the peroxisomal population in vivo, assuming that the particles differ from each other not only by size but also by protein composition (13, 19, 24, 30, 35, 39, 40). Our data reporting the possibility of temporal damage to the peroxisomal membrane followed by its resealing (see Broken peroxisomal membrane reseals) provides a new interpretation for the peroxisomal heterogeneity observed in vitro. Disruption of the membrane barrier results in the leakage of soluble matrix proteins through temporal “holes” in the membrane. One would expect that the rate of this leakage might be different and would depend on the molecular size of the protein. Consequently, smaller proteins escape from the particles, resulting in the formation of peroxisomal ghosts containing larger proteins. As seen in Fig. 8, the distribution of peroxisomal matrix proteins throughout the gradient clearly depends on their size and inversely correlates with the equilibrium density of the particles. For instance, SCP-2 (a monomeric protein of 12.5 kDa) is located in heavy (i.e., intact) and absent in light (i.e., peroxisomal ghosts) peroxisomes. Contrary to this, catalase (a tetrameric protein of 240 kDa) is present throughout the gradient, i.e., in intact and in damaged particles. Proteins like L-α-hydroxyacid oxidase (dimer of ~100 kDa), thiolase (dimer of 80 kDa), and SCP-2/thiolase (two dimeric forms: 104 and 116 kDa, respectively) occupy intermediate positions in the gradient.

![Fig. 9. Size-dependent leakage of matrix proteins from broken peroxisomes.](http://ajpcell.physiology.org/)

Sonication of intact peroxisomes isolated by means of an Optiprep gradient centrifugation. Peroxisomes were subjected to two cycles of sonication as described under MATERIALS AND METHODS. Immunodetection of peroxisomal proteins was performed by using J1 whole peroxisomal fraction or 2) supernatant obtained after centrifugation of sonicated peroxisomes at 100,000 g max for 60 min. Note the elevated rate of thiolase solubilization compared with catalase. PMP 22, indicating the location of the peroxisomal membrane, was found only in the sediment fraction. B–E: comparison of the peroxisomal fractions purified by means of the conventional technique or the new procedure (also see the legend to Fig. 7, C and D). B: enzyme composition of purified peroxisomes. The particles were diluted with isolation medium-2 to ensure equal activity in both peroxisomal preparations of urate oxidase (nucleoid marker) as an indicator of the “insoluble” part of peroxisomes. Data represent the total enzyme activity in the peroxisomal fraction isolated by the conventional procedure relative to the new method (100%). 1, urate oxidase; 2, catalase; 3, L-α-hydroxyacid oxidase; 4, lactate dehydrogenase; 5, thiolase. Note the difference in the enzyme composition between peroxisomal preparations. C and D: immunodetection of proteins in peroxisomes fractions isolated by the new procedure (lane 1) or by the conventional technique (lane 2). Samples with equal protein content were used. The immunosignal reflecting the position of SCP-2 on line 2 (C) was only barely detectable. Note the equal appearance of PM22 immunosignals, indicating similar content of peroxisomal membranes in both preparations. In contrast, the relative quantity of matrix proteins showed clear differences, with a higher proportion of large proteins in peroxisomes isolated by the conventional technique (see, i.e., catalase band). However, the content of small proteins in this preparation was much lower than in peroxisomes isolated by means of the new procedure (see, i.e., SCP-2 band). Matrix proteins containing a peroxisome targeting signal (PTS) 1 consensus sequence (–SKL) were immunodetected using anti-SKL antibodies (see D). The M, of marker proteins is indicated on left in D and E. The asterisks in D indicate the different protein contents in the peroxisomal preparations studied. Note enrichment in the SKL-containing proteins in peroxisomes isolated by the new procedure. E: silver staining of peroxisomal proteins separated by SDS-PAGE. Peroxisomes were isolated by the new procedure (lane 1) or by the conventional technique (lane 2). Samples with equal activity of urate oxidase were used for electrophoresis. Note the similarity in the density of the 34-kDa band corresponding to the monomer of urate oxidase in contrast to the clear difference in the density of some other protein bands (marked by asterisks), indicating enrichment of peroxisomes isolated by the new procedure in soluble matrix proteins.
To confirm this observation, we exposed intact peroxisomes isolated by means of an Optiprep gradient (fractions 4–7, see Fig. 8A) to two cycles of sonication. The organelles were then sedimented, and the rate of the leakage of different proteins from the particles was studied. As expected, the level of leakage was specific to each protein and inversely depended on their molecular size. Enzyme activity measurements revealed solubilization of 64.4 ± 2.6% l-α-hydroxyacid oxidase, 60.8 ± 3.4% lactate dehydrogenase, but only 38.4 ± 2.9% catalase from broken peroxisomes. These data were confirmed by immunodetection of the leakage of peroxisomal catalase and thiolase (Fig. 9A).

We then compared the protein composition of peroxisomes purified by the conventional technique and by the new method exploiting PEG 1500 as an osmoprotector (see Fig. 7, D and C, for a morphological assessment of purified peroxisomes). Analysis of enzyme activity showed a clear difference between the two peroxisomal preparations (Fig. 9B). Peroxisomes isolated by the conventional technique were low in some enzyme activities tested, especially thiolase. Comparative immunoblot analysis of both fractions (Fig. 9C) showed an elevated level of “small” proteins (thiolase, SCP-2) in peroxisomes isolated by means of the new procedure relative to particles purified by the conventional technique. Interestingly, peroxisomes isolated by the conventional method showed no detectable SCP-2 (see Fig. 9C, right). The protein composition of peroxisomes was further evaluated with antibodies to the consensus PTS 1 motif, serine-lysine-leucine (anti-SKL antibodies; Fig. 9D). These antibodies are capable of recognizing several peroxisomal matrix proteins (14). Peroxisomes isolated by the new procedure were enriched with some of these proteins (see Fig. 9D, left, marked by asterisks) while at least one of the detected proteins (asterisk in Fig. 9D, right) was more abundant in peroxisomes purified by the conventional method. SDS-PAGE revealed the appearance of additional protein bands in samples of peroxisomes purified by the new method (Fig. 9E).

**DISCUSSION**

Two main conclusions can be drawn from the results described here: 1) mammalian peroxisomes are osmotically sensitive particles and suffer an osmotic lysis when inappropriate conditions for their isolation are exploited; and 2) rupture of the peroxisomal membrane is a reversible process accompanied by leakage of soluble matrix proteins and the formation of peroxisomal ghosts. Importantly, resealing of the membrane leads to a restoration of its permeability properties. This observation implies that the widely employed technique of measuring of catalase latency is useless for the estimation of the integrity of purified peroxisomes.

The intraorganellar water balance may depend on the presence of: 1) small inorganic ions (e.g., Na⁺, Cl⁻, phosphate); 2) large, charged macromolecules (e.g., proteins) that attract small counterions; and 3) intermediate metabolites (amino acids, sugars, etc.), including cofactors and other relatively bulky organic molecules. All of these factors contribute to the intraorganellar osmolarity if the boundary membrane of the corresponding organelle is impermeable to the solute molecules (1).

The mammalian peroxisomal membrane allows free movement in and out of the particles to inorganic ions and small metabolites while restricting permeation to cofactors and other bulky organic molecules (Antonenkov VD, unpublished observation). As a consequence, one can expect that the intraperoxisomal osmolarity is determined in vitro only by the content in the particles of bulky organic molecules such as cofactors and ATP and does not depend on the presence of other solutes (small metabolites and unbound inorganic ions; Fig. 10A; we do not discuss here osmolarity caused by intraperoxisomal proteins).

Our data indicate that the behavior of peroxisomes during tissue homogenization and subcellular fractionation (in the absence of appropriate osmoprotectors) follows the steps shown in Fig. 10B. The imbalance in the concentration of compounds that determine peroxisomal osmolarity leads to the appearance of an osmotic pressure in the particles that provokes rupture of the membrane and leakage of some matrix constituents. The membrane rupture is temporal. Resealing of the membrane results in the formation of peroxisomal ghosts that have a low content of matrix constituents and, as a
consequence, are less vulnerable to osmotic disturbances. Resealing of the lipid bilayer after disruption caused by mechanical or osmotic treatments is a common feature of most biological membranes, including the plasma membrane of red blood cells (formation of erythrocyte ghosts), the inner bacterial membrane, the endoplasmic reticulum (formation of microsomes), and the outer mitochondrial membrane. Interestingly, the observation that the peroxisomal membrane restores permeability characteristics after resealing may be useful in simplifying studies on the mechanisms of transmembrane traffic of small metabolites and proteins by exploiting peroxisomal ghosts instead of freshly prepared, intact particles.

The precise dynamic picture of the formation of peroxisomal ghosts is not clear. Our data favor a model in which an osmotic pressure provokes formation of temporal splits in the membrane that allow soluble matrix constituents, including proteins, to escape out of the particles. This model may explain the observed dependence of the level of protein leakage from peroxisomes on their molecular size. It is reasonable to assume that an underestimation of the osmotic sensitivity of peroxisomes may lead to artificial observations not only in cell fractionation experiments (as described in the present study) but also in a broader spectrum of methodological approaches, including cell permeabilization. In addition, the difference in the leakage rate for soluble matrix proteins depending on their size compromises the use of typical peroxisomal marker catalase (large oligomeric protein) to determine dual localization of proteins of interest in peroxisomes and in the cytoplasm.

In summary, our results indicate that the behavior of mammalian peroxisomes in vitro is determined by unusual permeability properties of their membrane. The particles are osmotically sensitive in low-molecular-mass solutes. Their disruption by osmosis or damaging treatments (sonication, freezing) is accompanied by a temporal rupture of the membrane followed by its resealing and formation of peroxisomal ghosts containing less matrix proteins relative to intact organelles. The level of the leakage of these proteins through the broken membrane inversely depends on their molecular size. Disruption of peroxisomes during isolation can be partially prevented by applying appropriate osmoprotectors. On the basis of the data obtained, a new method for the isolation of nearly intact mammalian peroxisomes has been developed.

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