Isolation and characterization of membranes from oleic acid-induced peroxisomes of *Candida tropicalis*

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**Summary**

We report a methodology for the isolation of peroxisome membranes from the yeast *Candida tropicalis* pK233 grown on oleic acid, and the characterization of the polypeptide and lipid compositions of these membranes. Peroxisomes purified in either sucrose or Nycodenz gradients are treated with Tris-HCl (pH 8.5) and then with sodium carbonate (pH 11.5) to yield a final peroxisome membrane preparation (hereafter called ‘peroxisome membranes’). Electron microscopy revealed peroxisome membranes that are approximately 8.1 nm thick, have a typical trilaminar appearance, and form either flattened sheets or whorled structures. Peroxisome membranes contain 3.1 % and 2.2 % of the total protein of sucrose- and Nycodenz-gradient-purified peroxisomes, respectively. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis showed three predominant polypeptide bands of 34 (PMP 34), 29 (PMP 29), and 24 (PMP 24) × 10^3 M, in peroxisome membranes. Immunoblotting with an antiserum to PMP 24 showed that PMP 24 segregates with the peroxisome membrane fractions and is induced by growth of *Candida tropicalis* on oleic acid. Peroxisome membranes contain neutral lipids and phospholipids. The principal phospholipids are phosphatidyl choline and phosphatidyl ethanolamine. The phospholipid/protein ratio of peroxisome membranes is approximately 430 nmol mg⁻¹.

**Key words:** peroxisome, membrane, yeast.

**Introduction**

The peroxisome is surrounded by a single unit membrane. It is this membrane that maintains the integrity of the peroxisome, separating the peroxisome matrix from the cytosol and defining the interior of the peroxisome as a distinct intracellular space. Knowledge of the composition of the peroxisome membrane is essential for an understanding of peroxisome function and biogenesis.

The rat liver peroxisome membrane has been extensively studied. It has been characterized with regard to both its phospholipid and polypeptide compositions under normal conditions (Fujiki *et al.* 1982a) and its polypeptide composition under various conditions of peroxisome proliferation (Fujiki *et al.* 1984; Hashimoto *et al.* 1986; Hartl and Just, 1987). A number of polypeptides of the rat liver peroxisome membrane have been synthesized *in vitro*. These polypeptides are synthesized on free cytoplasmic polyribosomes, apparently without precursor extensions (Fujiki *et al.* 1984; Koster *et al.* 1986; Suzuki *et al.* 1987). The mechanism by which these polypeptides eventually become incorporated into the peroxisome membrane is not known.

An organism that has been used extensively as an experimental system for the study of peroxisome biogenesis is the yeast *Candida tropicalis* pK233. One powerful advantage of *C. tropicalis* for the study of peroxisome biogenesis is that peroxisome proliferation can be strikingly induced by growth of *C. tropicalis* on alkanes or fatty acids (Osumi *et al.* 1974, 1975; Tanaka *et al.* 1982). Peroxisomes from *C. tropicalis* contain catalase (Taranishi *et al.* 1974) and the enzyme system for the β-oxidation of fatty acids (Kawamoto *et al.* 1978; Dommes *et al.* 1983), a situation similar to that found in rat liver peroxisomes (Lazarow and de Duve, 1976; Lazarow, 1978; Hashimoto, 1982). Catalase and two of the enzymes of the β-oxidation system, which are localized to the peroxisome matrix, have been shown to be synthesized on cytoplasmic polyribosomes without precursor extensions (Yamada *et al.* 1982; Fujiki *et al.* 1986).

At this time, peroxisome membranes of *C. tropicalis* have not been isolated, and nothing is yet known about their composition. A knowledge of the composition of the peroxisome membrane is necessary for a complete picture of the phenomenon of peroxisome biogenesis in *C. tropicalis* as well as for an understanding of the metabolic functions of these peroxisomes. In this paper we report a method for the isolation of membranes from peroxisomes of *C. tropicalis*, and the characterization of the polypeptide and lipid compositions of these membranes.
Cultivation of yeast and isolation of peroxisomes

*Candida tropicalis* (Castellanii) Berkloout strain pK233 (ATCC 20336) (Tanabe et al. 1966) was grown at 30°C for 20 h on oleic acid-containing medium or for 10 h on glucose-containing medium (Kamiryo et al. 1982). Conversion of cells to spheroplasts, homogenization and preparation of a crude mitochondrial fraction were as described previously (Fujiki et al. 1986). Peroxisomes were purified from this crude mitochondrial fraction by equilibrium density centrifugation in a discontinuous sucrose density gradient (Kamiryo et al. 1982) or in a discontinuous Nycodenz density gradient (Small et al. 1987), except that centrifugation was performed in a Beckman VTi50 rotor at 100 000 g for 1 h. Fractions were analyzed for density (Fujiki et al. 1986), catalase (Baudhuin et al. 20336) (Tanabe et al. 1986), and cytochrome oxidase (Leighton et al. 1968).

Isolation of peroxisome membranes

Peak fractions of purified peroxisomes were adjusted to 30 mM Tris–HCl by addition of 1 M Tris–HCl (pH 8.5) and incubated on ice for 48 h. The peroxisomes were then diluted with 2 volumes of 10 mM Tris–HCl (pH 8.0), and a 'Tris membrane' pellet was isolated by centrifugation at 12 500 g for 2 h in a Beckman type 65 rotor at 4°C. The supernatant was decanted as the 'Tris supernatant', and the Tris membrane pellet was resuspended in 10 mM Tris–HCl (pH 8.0). The resuspended Tris membrane pellet was diluted with 200 volumes of ice-cold Na2CO3 (pH 11.5) (Fujiki et al. 1982b) and was incubated on ice for 2 h. The final peroxisome membrane preparation (hereafter called 'peroxisome membranes') was obtained by centrifugation at 200 000 g in a Beckman 50.2 Ti rotor for 2 h at 4°C. The supernatant was decanted as the 'sodium carbonate supernatant'.

Preparation of antisera to PMP 24 and to catalase and immunoblotting

PMP 24 (peroxisome membrane polypeptide 24) and catalase were isolated from peroxisome membranes and peroxisomes, respectively, by preparative sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (Fujiki et al. 1984). Antisera were raised by injection of the poly peptides into the popliteal lymph nodes of rabbits (Goudie et al. 1966; Fujiki et al. 1984). Immunoblotting was performed as previously described (Bur nette, 1981; Fujiki et al. 1984). Antibodies were visualized by incubation with 125I-labeled protein A followed by autoradiography. Lysates of *C. tropicalis* cells used in immunoblotting were prepared by glass bead disruption of intact cells (Needleman and Tzagoloff, 1975) in 50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 0.1 mM ZnCl2 and 1 mM phenylmethylsulfonyl fluoride.

Lipid analyses of peroxisome membranes

Lipids were extracted into chloroform–methanol by the procedure of Bligh and Dyer (1959). Lipid composition was assayed by one-dimensional thin-layer silica gel chromatography. Phospholipids were resolved in chloroform/methanol/water (65:25:4; by vol.) (Mangold, 1961) or in chloroform/methanol/25% ammonia (50:25:6; by vol.) (Sperka-Gottlieb et al. 1988). Neutral lipids were resolved in petroleum ether (50–110°C)/ethyl ether/acetec acid (80:20:1; by vol.) (Mangold, 1969). Phospholipids and neutral lipids were visualized by exposure of chromatography plates to iodine vapor (Marinetti, 1964). Sterols and sterol esters were detected by spraying chromatography plates with sulfuric acid/acetic acid (1:1, v/v), followed by heating at 90°C for 15 min (Jatzkewitz and Mehl, 1960). Phospholipid quantification was by the procedure of Bartlett (1959). Results are expressed as nanomoles of inorganic phosphate.

Analytical methods

Protein was determined by the method of Bradford (1976) with ovalbumin as the standard. SDS–polyacrylamide gel electrophoresis was performed as described (Fujiki et al. 1982b). Peroxisomes freshly isolated in sucrose density gradients and the 'Tris membrane and peroxisome membrane fractions were prepared for electron microscopy as previously described (Fujiki et al. 1982a).

Materials

SDS–polyacrylamide electrophoresis standards and lipid standards were from Sigma Chemical Co. (St Louis, MO) and NuChek-Prep (Elysian, MN). Silica gel G plates were from Analtech Inc. (Newark, DE). 125I-labeled protein A was from Amersham (Oakville, ON). Nycodenz centrifugation medium was from Accurate Chemical and Scientific Corp. (Westbury, NY). All other chemicals and materials were of the highest grade commercially available.

Results

Isolation of peroxisomes and peroxisome membranes

Peroxisomes were purified by differential centrifugation followed by equilibrium density centrifugation in gradients of sucrose or Nycodenz. Representative fractionations of such gradients are shown in Fig. 1A for sucrose and in Fig. 1B for Nycodenz. Peroxisomes (catalase as the marker enzyme) and mitochondria (cytochrome oxidase as the marker enzyme) were well separated from one another in both gradient systems. In sucrose density gradients, peroxisomes sedimented to a symmetrical peak with an equilibrium density of 1.23 g cm⁻³, whereas mitochondria had a density of 1.17 g cm⁻³ (Fig. 1A). In Nycodenz density gradients, peroxisomes had an equilibrium density of 1.19 g cm⁻³, while mitochondria had a density of 1.11 g cm⁻³ (Fig. 1B). The peak peroxisome fraction contained 2.0% of the cytochrome oxidase present in the gradient in the case of sucrose and less than 0.1% in the case of Nycodenz.

An electron micrograph of peroxisomes purified on a sucrose density gradient is shown in Fig. 2A. Only rarely was a mitochondrial profile observed. Treatment of peroxisomes with Tris–HCl (pH 8.5) yielded a Tris membrane pellet (Fig. 2B). Most of the peroxisomes in the Tris membrane pellet appeared opened and vacuolated, although some granular matrix material was still apparent. Long continuous membrane sheets were also apparent. Treatment of the Tris membrane pellet with sodium carbonate (pH 11.5) to yield the peroxisome membrane pellet released the remaining matrix material and converted most of the membranes into long sheets, although some small, circular membrane structures were evident (Fig. 2C). The peroxisome membranes retained their trilaminar appearance (Fig. 2C, inset) and had a thickness of 8.1±1.0 nm as determined from several measurements made directly from the micrographs.

Polyepitide composition of peroxisome membranes

SDS–gel electrophoretic analysis of peroxisomes and of
the soluble and membrane subfractions is seen in Fig. 3. Most of the catalase (open arrowhead), the two forms of fatty acyl-CoA oxidase, POX 2 and POX 4 (filled arrowheads) and hydratase–dehydrogenase–epimerase (double arrowhead) (Fujiki et al. 1986) were liberated to the soluble Tris supernatant subfraction by treatment of peroxisomes with Tris–HCl (pH 8.5) (lane 2). The soluble proteins resistant to this procedure were removed from the Tris membrane pellet (lane 3) by treatment with sodium carbonate (arrows, lane 4). The peroxisome membrane pellet (lane 5) showed three major polypeptide bands having relative molecular masses of 33 900±200, 29 100±400 and 23 700±200 (means and standard deviations from 10 separate experiments),
Table 1. Protein distribution of peroxisome subfractions

<table>
<thead>
<tr>
<th>Subfraction</th>
<th>Sucrose</th>
<th>Nycodenz</th>
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<tbody>
<tr>
<td>Tris supernatant</td>
<td>3</td>
<td>550±27</td>
</tr>
<tr>
<td>Tris membranes</td>
<td>3</td>
<td>436±4</td>
</tr>
<tr>
<td>Carbonate supernatant</td>
<td>3</td>
<td>264±39</td>
</tr>
<tr>
<td>Peroxisome membranes</td>
<td>4</td>
<td>312±4</td>
</tr>
</tbody>
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The results are based on 1000 µg of starting peroxisomal protein. Recoveries are given in micrograms.

Table 1 shows the recoveries of protein for the peroxisome subfractions of peroxisomes isolated either in sucrose or in Nycodenz density gradients. Peroxisome membranes contained 3.1±0.4% and 2.2±0.3% of the total protein of peroxisomes isolated in sucrose and Nycodenz density gradients, respectively.

PMP 24 purifies with the membrane fractions of peroxisomes

Immunoblotting of peroxisome subfractions with anti-PMP 24 serum demonstrated the presence of small amounts of PMP 24 in whole peroxisomes (Fig. 4A, lane 1) and the segregation of PMP 24 to the membrane subfractions of peroxisomes (Fig. 4A, lanes 3 and 5). No PMP 24 was found in the soluble protein fractions (Fig. 4A, lanes 2 and 4). In contrast, the soluble protein catalase is found in whole peroxisomes (Fig. 4B, lanes 2 and 4). In the soluble Tris supernatant subfraction of peroxisomes (Fig. 4B, lane 1) and in the soluble Tris supernatant subfraction of peroxisomes (Fig. 4B, lane 2) but not in the membrane subfractions (Fig. 4B, lanes 3 and 5). Significantly, no breakdown products of either catalase or PMP 24 are observed after the lengthy incubations required during the isolation.

PMP 24 is induced by growth of C. tropicalis on oleic acid

Peroxisomes and peroxisome proteins are markedly induced by growth of C. tropicalis cells on oleic acid as opposed to growth on glucose (Fujiki et al. 1986). The induction of PMP 24 by growth of C. tropicalis on oleic acid was investigated by immunoblotting. Polypeptides from lysates of cells grown on oleic acid (Fig. 5A, lane 1) and glucose (Fig. 5A, lane 2) were separated by SDS–gel electrophoresis and probed with antisera to PMP 24 and to catalase. PMP 24 was induced by growth of cells on oleic acid versus growth on glucose (Fig. 5C, cf. lanes 1 and 2, respectively). There was also marked induction of catalase by growth of cells on oleic acid (Fig. 5B, cf. lanes 1 and 2, respectively).

Lipid composition of peroxisome membranes

The peroxisome membrane was shown by one-dimensional thin-layer chromatography to contain both phospholipid and neutral lipid. The major phospholipid constituents were phosphatidyl choline and phosphatidyl ethanolamine (Fig. 6A). No other phospholipid constituents were observed even when excess amounts of material were analyzed (data not shown). By this analysis, the
Fig. 4. Segregation of PMP 24 and catalase with peroxisome subfractions. Peroxisomes isolated in a Nycodenz density gradient and subfractions from a peroxisome membrane isolation were analyzed by SDS–polyacrylamide gel electrophoresis followed by transfer to nitrocellulose. A 25 µg sample of protein was loaded in each lane, except in A, lane 1 (100 µg). Lane 1, peroxisomes. Lane 2, soluble proteins from Tris supernatant. Lane 3, Tris membranes. Lane 4, soluble proteins from sodium carbonate supernatant. Lane 5, peroxisome membranes. The polypeptides were probed with a polyclonal antiserum to PMP 24 at 1:700 dilution (A) or to catalase at 1:300 dilution (B). Bound antibody was detected by incubation with I-125-labeled protein A followed by exposure to Kodak XAR film at -70°C with one Dupont Cronex Lightning Plus intensifying screen for 11 days (A, lane 1), 6 days (A, lanes 2–5) or 3 days (B).

Discussion

In this paper we have developed an approach using a combination of Tris–HCl and sodium carbonate extraction of gradient-purified peroxisomes of the yeast C. tropicalis pK233 grown on oleic acid for the isolation of peroxisome membranes. Attempts at isolating peroxisome membranes by standard procedures such as treatment with sodium carbonate alone or treatment with Tris–HCl alone were unsuccessful, yielding inconsistent results and high contamination of peroxisome membranes with soluble peroxisome proteins. Shorter incubation times in Tris–HCl (1 h, 6 h or 24 h) were also found to lead to an inefficient separation of the proteins in the soluble and membrane fractions (data not shown). The reasons for the failure of these techniques to give clean peroxisome membranes are unknown. The procedure we describe in this paper reproducibly yields clean peroxisome membranes, is nondestructive (soluble+membrane proteins=starting proteins) and efficient (polypeptide bands are either in soluble fractions or in the final membrane pellet, but not both).

The percentage of total protein present in the peroxisome membrane of C. tropicalis (3.1±0.4 % and 2.2±0.3 % for sucrose and Nycodenz peroxisomes, respectively) is lower than the percentage of total protein reported for membranes of mammalian liver peroxisomes (between 6 and 29 %). The major polypeptides of the peroxisome membrane of C. tropicalis (PMPs 34, 29 and 24) are different from those of mammalian liver peroxisomes (a doublet at approximately 69K and 22K (Fujiki et al. 1982a; Crane et al. 1985; Hartl and Just, 1987). The PMPs of the olate-induced peroxisomes of C. tropicalis are significantly different from those of the methanol-induced PMPs of Candida boidinii (Goodman et al. 1986), the only other yeast peroxisome membranes yet isolated. The PMPs of C. boidinii show two prominent polypeptides of 47 and 20K and three less-abundant...
Fig. 5. Induction of catalase and PMP 24 by growth of C. tropicalis on oleic acid. A 150 µg sample of protein from lysates of C. tropicalis grown on oleic acid (lane 1) or on glucose (lane 2) was separated by SDS–polyacrylamide gel electrophoresis followed by transfer to nitrocellulose. A. Coomassie Blue-stained polypeptides. The polypeptides were probed with a polyclonal antiserum to catalase at 1:300 dilution (B) or to PMP 24 at 1:700 dilution (C). Bound antibody was detected by incubation with 125I-labeled protein A followed by exposure to Kodak XAR film at —70 °C with one Dupont Cronex Lightning Plus intensifying screen for 16 h (B) or 5 days (C). Numbers at the left and right represent the positions of molecular weight standards as in Fig. 3.

Polypeptides of 120, 32 and 31K by one-dimensional SDS–polyacrylamide gel electrophoresis. As has been demonstrated for a number of soluble peroxisomal proteins of C. tropicalis (Fujiki et al. 1986), there is an induction of PMP 24 by growth of C. tropicalis on oleic acid as opposed to glucose.

The increased amount of PMP 29 in membranes of peroxisomes isolated in sucrose density gradients compared to those from peroxisomes isolated in Nycodenz density gradients (Fig. 3, cf. lanes 5 and 6, respectively) may be due to a contaminating polypeptide of approximately 30K from mitochondrial membranes. We do not believe that this difference in amounts of PMP 29 is due to differential extraction of PMP 29 from membranes of peroxisomes isolated in sucrose as opposed to Nycodenz, since no PMP 29 is seen in the soluble fractions during the isolation. There is greater contamination by mitochondria in peroxisomes isolated in sucrose gradients than those isolated in Nycodenz gradients and a 30K polypeptide is the major membrane polypeptide of mitochondria of C. tropicalis (data not shown). However, the amount of PMP 29 in final membranes of peroxisomes isolated on Nycodenz density gradients, coupled with the almost total absence of any mitochondrial contamination of peroxisomes isolated on Nycodenz density gradients, suggests that PMP 29 is a bona fide peroxisome membrane polypeptide of 29K.
The thickness of the peroxisome membrane of *C. tropicalis* (8.1±1.0 nm) is comparable to that of rat liver peroxisome membranes (6.8±0.8 nm) (Fujiki et al. 1982a). The *C. tropicalis* peroxisome membrane is composed of phospholipids and neutral lipids, including sterols. As in rat liver peroxisome membranes, the major phospholipid components of peroxisome membranes of *C. tropicalis* are phosphatidyl choline and phosphatidyl ethanolamine. No cardiolipin, a characteristic phospholipid of mitochondria, was found in peroxisome membranes of *C. tropicalis*. No information on the lipid composition of peroxisome membranes of other yeasts is available for comparison. Compared to rat liver peroxisome membranes (Fujiki et al. 1982a), there is approximately twice as much phospholipid to protein in *C. tropicalis* peroxisome membranes (200 as compared to 430 nmol mg protein⁻¹, respectively).

The proteins in the peroxisome membrane may play distinct roles in maintenance of the structure of the peroxisome, in the import of proteins into the peroxisome, in the transport of molecules across the peroxisome membrane, and in the generation of an electrical potential across the peroxisome membrane. What roles the peroxisome membrane proteins identified here play in the biogenesis and biochemistry of peroxisomes in *C. tropicalis* remain to be determined.

We thank Dr David W. Andrews and Dr Karl B. Freeman for critical reading of this manuscript and helpful discussion. We also thank Mr Remo Bottega for help with the lipid analyses. This work was supported by grant MA-9208 from the Medical Research Council of Canada to R.A.R., who was supported by a scholarship from the MRC. A.G.B. was supported by a studentship from the MRC.

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(Received 2 October 1989 - Accepted 11 December 1989)