Polypeptide and Phospholipid Composition of the Membrane of Rat Liver Peroxisomes: Comparison with Endoplasmic Reticulum and Mitochondrial Membranes

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ABSTRACT Membranes were isolated from highly purified peroxisomes, mitochondria, and rough and smooth microsomes of rat liver by the one-step Na₂CO₃ procedure described in the accompanying paper (1982, J. Cell Biol. 93:97-102). The polypeptide compositions of these membranes were determined by SDS PAGE and found to be greatly dissimilar. The peroxisomal membrane contains 12% of the peroxisomal protein and consists of three major polypeptides (21,700, 67,700 and 69,700 daltons) as well as some minor polypeptides. The major peroxisomal membrane proteins as well as most of the minor ones are absent from the endoplasmic reticulum (ER). Conversely, most ER proteins are absent from peroxisomes.

By electron microscopy, purified peroxisomal membranes are ~6.8 nm thick and have a typical trilaminar appearance. The phospholipid/protein ratio of peroxisomal membranes is ~200 nmol/mg; the principal phospholipids are phosphatidyl choline and phosphatidyl ethanolamine, as in ER and mitochondrial membranes. In contrast to the mitochondria, peroxisomal membranes contain no cardiolipin. All the membranes investigated contain a polypeptide band with a molecular mass of ~15,000 daltons. Whether this represents an exceptional common membrane protein or a coincidence is unknown. The implications of these results for the biogenesis of peroxisomes are discussed.

Knowledge of the peroxisomal membrane's properties is essential to an understanding both of the organelle's functions and of its biogenesis. The membrane separates the peroxisomal contents from the cytosol and defines the peroxisomal interior as a distinct intracellular space. The permeability properties of the membrane determine to what extent the peroxisome functions as a separate biochemical compartment. Knowledge of how the membrane is formed is essential to an understanding of the biogenesis of the organelle as a whole. If the membrane is derived from some other intracellular membrane, for example the endoplasmic reticulum (ER) (as is widely assumed), then one might expect to see some similarity in composition between them. If, on the other hand, the peroxisomes exist as a separate intracellular compartment, as has recently been suggested (1), then the peroxisomal membrane needs to have no structural similarity to the ER.

We have applied the newly-developed sodium carbonate procedure described in the accompanying paper (2) to isolate peroxisomal, mitochondrial, and ER membranes. We have partially characterized these three membranes, and found that their polypeptide compositions are almost entirely different, but their phospholipid compositions are similar. Some of these results have appeared in abstract form (3, 4).

1 Rat liver microsomes were subfractionated by isopycnic centrifugation in linear sucrose gradients according to Beaufay et al. (7). The fractions selected as the "rough microsomal fraction" have been shown by these authors to consist almost exclusively of vesicles derived from the rough endoplasmic reticulum. This justifies the use of the term "endoplasmic reticulum membranes" rather than the operational expression "microsomal membranes."
MATERIALS AND METHODS

Preparation of Membranes by Means of Sodium Carbonate Treatment

Peroxisomes or other organelles were diluted 100-fold with ice-cold 100 mM Na₂CO₃, pH 11.5, kept on ice for 30 min, and centrifuged for 1 h at 50,000 rpm in a Beckman 50 Ti rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) as described previously (2).

Isolation of Organelles

Peroxisomes were purified from rat liver by sequential differential and equilibrium density centrifugation exactly as described by Leighton et al. (5). The purity of the peroxisomes was determined by measurement of specific marker enzymes for the various organelles. We selected the three purest peroxisome preparations of 14 that were prepared, and used fractions on the dense side of the peroxisome peak to further minimize contamination by other, less dense organelles. As shown in Table I, the relative specific activity of catalase in the purified peroxisomes was 35, which in comparison to the results of Leighton et al. (5) implies ~93% purity. The ratio of catalase/cytochrome oxidase (a mitochondrial marker) is 2,500, and the ratio of catalase/esterase (a microsomal marker) is 65. From the relative specific activities in Table I and the fact that mitochondria and ER each contributes ~20% of total liver protein (5-7), we calculate that the contamination of peroxisomes by mitochondria is 0.040 × 20 = 0.8%, and the contamination by microsomes is 0.175 × 20 = 3.5%. Free peroxisome cores, which are known to be concentrated on the dense side of the peroxisome peak (5), probably contribute the bulk of the remaining protein. It should be emphasized that microsomes are a common contaminant of peroxisomes (because rough microsomes have a density similar to that of peroxisomes), and without the precautions taken here, they may constitute a considerable portion of the protein present.

Mitochondria were also purified by sequential differential and isopycnic centrifugation (5) and were ~86% to 93% pure. The preparation of rough and smooth microsomal subfractions by centrifugation in linear sucrose gradients and centrifugation (5) and were 86% to 93% pure. The preparation of rough and smooth microsomal subfractions by centrifugation in linear sucrose gradients and centrifugation (5) and were 86% to 93% pure.

Analytical Methods

SDS PAGE, electron microscopy of membranes, enzyme assays, and protein determinations were carried out as described (2, 5). Freshly isolated peroxisomes were prepared for electron microscopy according to Baudhuin et al. (9) but with modifications (5) to prevent osmotic lysis during fixation. The fixed peroxisomes were collected by filtration on VC Millipore filters (0.1-μm pore size; Millipore Corp., Bedford, MA).

Lipids were extracted into chloroform-methanol by the procedure of Bligh and Dyer (10). For quantification, aliquots of the chloroform phase were dried down and assayed for organic phosphate after Mg(NO₃)₂ ashing (11). The results are expressed in nanomoles of organic phosphate. Phospholipid compositions were measured by chromatography on 250-μm thick Silica Gel G plates using chloroform/methanol/water (65:25:4, vol/vol) as solvent (12). After chromatography the plates were sprayed with 55% H₂SO₄ (wt/vol) containing 0.6% K₂CrO₄ (wt/vol) and charred at 180°C for 15 min in order to detect the lipids.

Materials

Thin layer chromatography plates (Prekotes, Silica Gel G) were purchased from Applied Science Div., Milton Roy Co., Laboratories Group (State College, PA). Phospholipid standards were from Supelco, Inc. (Bellefonte, PA). Other materials were obtained as before (2).

RESULTS

Characterization of Peroxisomal Membranes

Peroxisomes (Fig. 1a) were treated with 100 mM sodium carbonate and centrifuged. Electron microscope examination of the pellet revealed membranes, mostly in the form of unsealed fragments with sizes of ~0.1 to 0.5 μm (Fig. 1b). These included the major peroxisosomal protein catalase, located in the organelle matrix, and urate oxidase, located in the core. The Na₂CO₃ dissolved the cores (13, 14), and quantitatively released the matrix proteins. One polypeptide band, visible among the total peroxisomal proteins (indicated with an arrow in Fig. 2), was present only in the membranes and not among the soluble proteins. Two other membrane polypeptides (arrowheads) could not be discerned among the total proteins due to the large amount of soluble proteins in this region of the gel. The sum of the membrane and soluble components (Fig. 2, lanes B and C) was similar to the starting material (Fig. 2, lane A), indicating apparently quantitative recovery. The smaller membrane polypeptide consistently appeared as a sharp band in SDS PAGE and has been observed in all of our membrane preparations. The two larger membrane polypeptides varied in their appearance; occasionally they were somewhat fuzzy or not resolved from one another in SDS PAGE (Fig. 6, lanes 2 and 6). The same pattern of membrane proteins as that shown in Fig. 2 was observed when peroxisomal membranes were isolated in the presence of a mixture of protease inhibitors (not illustrated). The pattern was unaffected by a second extraction with carbonate.

The molecular masses of the peroxisomal membrane polypeptides were estimated by comparison with known standards in SDS PAGE. The three prominent bands have mean masses of 69,700 ± 1,300, 67,700 ± 1,000, and 21,700 ± 300 daltons (means and standard deviations of 12 determinations). Peroxisomal membranes contained ~12% of the total peroxisomal proteins (Table II). The membranes were highly enriched in phospholipids in comparison to the released material. The phospholipid/protein ratio was 204 nmol phosphate/mg membrane protein (Table II). This is ~60% of the phospholipid/protein ratio of 340 ± 40 nmol/mg that we found for two preparations of microsomal membranes (not shown).

Characterization of Mitochondrial Membranes

Application of the carbonate procedure to purified mitochondria yielded membranes (Fig. 3) which contained ~21% of total mitochondrial proteins (Table III). SDS PAGE analysis revealed about a dozen major membrane polypeptides, as well
as many minor ones (Fig. 4). These are presumably a mixture of inner and outer membrane proteins.

**Comparison of the Polypeptide Composition of Peroxisomal and Mitochondrial Membranes**

The final step in the purification of peroxisomes and mitochondria is centrifugation in a sucrose gradient. When membranes were prepared from each fraction of the gradient (by exposure to Na$_2$CO$_3$), the three major peroxisomal membrane polypeptides were clearly visible, peaking in the peroxisomal region of the sucrose gradient (Fig. 5, lanes 3 and 4, arrows). These bands decreased in intensity as the density decreased into the mitochondrial region of the gradient. This demonstrates that these three polypeptides are true peroxisomal proteins and do not belong to contaminating mitochondria. The mitochondrial membrane proteins were observed at maximum concentration in fraction 8, but could still be detected in small amounts in the peak peroxisomal fraction (fraction 4). This illustrates the cross-contamination that occurs, and demonstrates the need to select fractions on the dense side of the peroxisomal peak to obtain sufficiently pure membranes.

**Comparison of the Polypeptide Composition of Peroxisomal, Mitochondrial, and ER Membranes**

Equal amounts of the membranes of the various highly purified organelles were analyzed by SDS PAGE (Fig. 6). The patterns of membrane polypeptides differed strikingly among peroxisomes, mitochondria, and microsomes, whereas the two subclasses of microsomal membranes were similar to each other. Cytochrome P-450 and other major microsomal proteins were absent from the peroxisomal membranes. Conversely, the three main peroxisomal membrane proteins were absent from the microsomal membranes. The mitochondrial pattern was also unique.

The amount of peroxisomal membrane protein analyzed was considerably larger in this experiment, and some minor bands were visible (Fig. 6, lanes 2 and 6). A few of these may be present owing to residual contamination by mitochondria and ER, others may represent traces of soluble peroxisomal proteins, and still others may be genuine constituents of the peroxisomal membrane.

Although the polypeptide compositions of the three organelles' membranes were distinctly different, a few polypeptides with the same apparent size were present in the three membranes in amounts too large to be attributed to cross-contamination. The most prominent of these (labeled with an asterisk) had a molecular mass of $\sim$15,000 daltons, and a similar intensity in all samples. Whether this represents a common membrane protein in all three organelles remains to be determined by methods other than one-dimensional SDS PAGE. In addition, both mitochondrial and peroxisomal membranes contained small amounts of a polypeptide that comigrated with microsomal cytochrome b$_5$ (Fig. 6). This is consistent with the presence of this cytochrome in outer (but not inner) mitochondrial membranes (15–17) and its reported presence in small amounts in the peroxisomal membrane (18).

The traces of cytochrome P-450 visible in these large samples of peroxisomal membranes can be entirely accounted for by the residual 3.5% contamination of the purified peroxisomes by endoplasmic reticulum (see Materials and Methods). This illustrates the limits of the methodologies and the need for quantitative evaluation of membrane purity.
Comparison of the Lipid Compositions of the Membranes

All membrane preparations investigated contained phosphatidyl choline and phosphatidyl ethanolamine as their major phospholipid constituents (Fig. 7). Cardiolipin was found only in the mitochondrial membranes. Some sphingomyelin may be present in microsomal fractions, possibly owing to the presence of plasma membranes in these preparations (19). Lyso derivatives of the phospholipids were evident in various amounts in different membrane preparations and were probably the result of endogenous phospholipases acting on the preparations during organelle isolation and storage.

All of the membrane fractions contained some cholesterol and cholesteryl ester. These appeared as rapidly migrating bands near the solvent front in the thin layer chromatograms shown in Fig. 7, and were identified in two other solvent systems (hexane:diethyl ether:acetic acid (80:20:1, vol/vol/vol) and isopropyl ether:glacial acetic acid (96:4, vol/vol), not illustrated). The amounts of cholesterol found were much smaller...
than what was present in plasma membranes (analyzed for comparison in Fig. 7, lane 8). It has been shown that most of the cholesterol in microsomal fractions is in contaminating plasma membranes (7, 8).

It is apparent from Fig. 7 that the relative abundances of the various lipids is not identical in all the membranes. Thus far, insufficient material has been obtained for chemical quantification.

Procedure

In several control experiments we found that the most important parameter for the successful isolation of peroxisomal and mitochondrial membranes was the pH, as it was for microsomal membranes (2). Adjusting the pH to 11 with 1 mM K$_2$B$_4$O$_7$ caused a release of proteins similar to that produced by 100 mM Na$_2$CO$_3$ (as judged by SDS PAGE, not illustrated). Lower pHs were less effective or ineffective. 250 mM NaHCO$_3$ or NaCl or KCl would not substitute for the carbonate. A second application of Na$_2$CO$_3$ to isolated peroxisomal membranes did not change the polypeptide composition.

DISCUSSION

Comparison of Membranes

In this investigation, we have isolated three intracellular membrane systems. We can calculate that they vary greatly in their abundance, ranging from 0.8 mg of peroxisomal membrane protein/g liver to 28 mg of ER membrane protein/g liver (Table IV). This disparity, and the similar densities of rough ER and peroxisomes, are the causes of the difficulty in

FIGURE 4 Preparation of mitochondrial membranes by sodium carbonate procedure; analysis by SDS PAGE. (A) Total mitochondrial protein—100 µg. Membrane (B) and soluble proteins (C) derived from 100 µg of mitochondrial protein. Total (D), membrane (E), and soluble proteins (F) from 200 µg of mitochondrial protein. STD, molecular weight standards.

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FIGURE 5 SDS PAGE analysis of membranes prepared from each fraction of a sucrose gradient used to separate peroxisomes and mitochondria. Membranes were prepared from 6-µl aliquots of the fractions (5-570 µg protein). Numbers at the top indicate fraction numbers, starting at the bottom of the gradient. Peroxisomes were mainly in fractions 3 and 4. Mitochondria were most abundant in fraction 8, but extended down as far as fraction 4. The three main peroxisomal membrane proteins are indicated with arrows. Two mitochondrial membrane proteins visible in lane 4 are indicated with dots. Standard proteins (STD) as in Fig. 2.

FIGURE 6 Polypeptide composition of organelle membranes. Equal amounts of each type of membrane (50 µg protein) were analyzed by SDS PAGE. Lanes 1 and 4, mitochondria; 2 and 6, peroxisomes; 3 and 7, rough microsomes stripped of ribosomes with pyrophosphate; 5 and 8, smooth microsomes. STD, molecular weight standards. Identification of cytochromes b$_5$ and P-450 as in reference 2. Asterisk indicates a stained band visible in all the membranes.
obtaining pure peroxisomal membranes.

SDS PAGE analysis (Fig. 6) revealed very different patterns for the three membrane types, indicating that (a) the three organelles contain mostly different proteins in their membranes and (b) the three types of membrane are each quite pure. A few similarities were noted, including the presence of a 15,000 dalton polypeptide in about the same abundance in all three membranes, and some cytochrome b2 in each. It is not known whether the 15,000 dalton band is the same polypeptide in the three membrane types. These results do not exclude the presence of other components or enzymes in trace amounts in all three membranes.

In contrast to the very different protein compositions, the phospholipid compositions of the various membranes were qualitatively similar, consisting largely of phosphatidyl choline and phosphatidyl ethanolamine. Cardiolipin was found only in mitochondria.

Some cholesterol was observed in the membrane preparations, especially in the microsomal fractions. The presence of cholesterol in microsomal fractions was described by Dallner and Ernster (20), but later Beaufay et al. (7) and Amar-Costeseq et al. (8) repeated that the bulk of this cholesterol was actually in fragments of the plasma membrane, which constitute ~7-8% of the protein of the microsomal fraction (7), and which are known to be very rich in cholesterol (21). Our own results are generally compatible with this view, except that qualitatively it appears that we have more cholesterol in the rough microsomal fraction than would be expected from the quantitative results of Beaufay et al. (7). The explanation may be that, to purify the peroxisomes, our rats were pre-treated with Triton WR-1339, which is known to produce a hypercholesterolemic serum (22, 23), as well as an accumulation of cholesterol in hepatic lysosomes (24). Since the livers were not perfused, cholesterol might have adsorbed onto the membranes during homogenization.

Cholesterol was also observed in the peroxisomal and mitochondrial membrane preparations (Fig. 7). Considering the various amounts of membrane protein analyzed (see legend to Fig. 7), we estimate that the cholesterol/protein ratio is of the order of 10 to 20 times lower in these preparations than in the purified plasma membranes. Whether this cholesterol is a true constituent of peroxisomal and mitochondrial membranes cannot be decided with certainty at present. It could be accounted for by a 5-10% contamination by plasma membranes, or could originate from the hypercholesterolemic serum.

**Methodology**

The carbonate procedure (2) has proved successful for the isolation of three types of endomembrane in this investigation. It may be emphasized that the method is nondestructive (soluble + membrane proteins = starting proteins) and efficient (polypeptide bands are generally found to be either soluble or in the membranes, but not both). As discussed by Fujiki et al. (2), the carbonate procedure appears to effectively release peripheral membrane proteins, and this conclusion is further corroborated by our results.

We have combined the use of the sodium carbonate procedure with isopycnic centrifugation and SDS PAGE to analyze membrane proteins as a function of their size and of the density of their host organelle (Fig. 5). This procedure may prove useful in other studies of cell architecture.

**The Peroxisomal Membrane**

The peroxisomal membrane contains 12% of the total peroxisomal protein, three major polypeptides (21,700, 67,700, and 69,700 daltons) and some minor polypeptides. None of its major proteins are present in the ER; conversely, the peroxisome lacks most ER proteins. The phospholipid/protein ratio of the peroxisomal membrane is ~200 nmol/mg; the major lipids are phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl serine (3).

3 Haja et al. (35) have demonstrated that the first enzyme in ether glycerolipid biosynthesis, acyl-CoA:diacylglycerol acyltransferase, is located in peroxisomes in rat liver. In the absence of any information on the comparative mobilities of ether glycerolipids and the usual phospholipids in the solvent system used, it is conceivable that some of the peroxisomal lipids are actually ether glycerolipids.
and perhaps cholesterol. Thus the phospholipid composition of the peroxisomal membrane is qualitatively similar to that of the ER but the phospholipid/protein ratio appears to be lower.

Our conclusions differ from those of Donaldson et al. (25), who emphasized the similarity of peroxisomal and ER membranes in rat liver. It would appear that this is due to contamination of their peroxisomal membranes with ER. Those authors report that the specific activity of glucose-6-phosphatase in their purified peroxisomes was 35% of the specific activity in purified microsomes (their Table VIII); this means that 35% of the protein in their purified peroxisomes was actually ER, because peroxisomes lack glucose-6-phosphatase altogether (5). Since the membrane represents a larger percentage of the total organelle protein in microsomes than in peroxisomes (53% vs. 12%), we calculate that ~70% of the membrane protein in the purified peroxisomes of Donaldson et al. (25) was derived from the ER. Under these conditions, it is not surprising that their "peroxisomal membranes" appeared similar to ER.

Peroxisome Biogenesis

Peroxisomes have long been thought to form by budding from the ER. This theory is based in large part on published morphological observations showing proximity as well as possible connections between peroxisomes and ER. Some investigators, especially Novikoff et al. (26), argue that connections between these two organelles are common, whereas other scientists report not finding any connections after careful search (27, 28), including serial sections (28). Recent experiments of Shio and Lazarow (29) found no diffusion of cytochemical reaction products between peroxisomes and ER, consistent with there being no connections. Novikoff et al. (26) have even suggested that small anucleate peroxisomes (referred to by them as "microperoxisomes") are "specialized regions of smooth ER," an idea disproven by our results (since we find that peroxisomes are not bounded by ER membranes).

Were the polypeptide composition of the peroxisomal membrane to resemble that of the ER, this would strongly support the budding hypothesis. However, such is not the case, and thus our results support and extend other biochemical investigations of the past 15 years that have similarly found no evidence for a role of the ER in peroxisome biogenesis (reviewed by Lazarow et al. [1]). For example, catalase, the principal matrix protein of the peroxisome, does not pass through the ER on its way to the peroxisomes (30-33).

If there are any connections between peroxisomes and ER, they must be such as to prevent catalase and other peroxisomal matrix proteins from diffusing into the ER. They must prevent serum albumin and other secretory proteins from entering peroxisomes, they do not allow peroxisomal membrane proteins to diffuse within the plane of the membrane into the ER membrane, and they do not allow ER membrane proteins to enter the peroxisomal membrane. In addition, any such connections play no role in the biogenesis of catalase.

It is possible that the peroxisomal membrane proteins are synthesized on bound polysomes and that the peroxisomal membrane could form from the ER by "capping" of peroxisomal membrane proteins within the plane of the ER membrane, followed by outpouching and pinching off. Such a process occurs in the formation of the envelope of VSV virus from plasma membrane (34). However, the viral core provides a matrix to which the viral envelope proteins may bind specifically as the virus buds out through the plasma membrane, and no such nucleation mechanism is available for the budding of the peroxisomal membrane (the peroxisomal core protein, urate oxidase, is synthesized on free polysomes [33]). Also, the remodeling of the peroxisomal membrane according to this hypothesis would have to be virtually total, since we detect practically no overlap between the polypeptide compositions of the ER and peroxisomal membranes.

Lazarow et al. (1) have recently pointed out that almost all of the biochemical data in the literature, and most of the morphological observations, fit a model in which peroxisomes exist in the cell without connections to the ER but with transient interconnections among themselves. It was suggested that newly synthesized peroxisomal constituents (soluble, core, and membrane components) could be added to pre-existing peroxisomes, and perhaps then be further distributed by fission and fusion events. Our results on the unique polypeptide composition of the peroxisomal membrane are compatible with this hypothesis.

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