Biochimica et Biophysica Acta, 945 (1988) 135–144
Elsevier

BBA 74197

Analysis of the major integral membrane proteins of peroxisomes from mouse liver

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(Received 26 April 1988)

Key words: Peroxisomal membrane; Integral membrane protein; Clofibrate; (Mouse liver)

Two major proteins with subunit molecular masses of 68 and 70 kDa were isolated from the integral membrane protein fraction of peroxisomes purified from mouse liver. The two proteins were shown to be distinct proteins by two criteria: first, immunoblot analysis demonstrated that antisera against the 68 kDa protein did not cross-react with the 70 kDa protein, and vice versa; and second, the partial peptide maps resulting from proteinase digestion of the proteins were different. Immunoblot analyses to test the specificities of the antisera demonstrated that only the expected molecular mass species in purified peroxisomes, and in membranes prepared from these organelles, were recognized; there was no identification of proteins from purified mitochondrial or microsomal fractions. The concentrations of both of these proteins were increased in livers of mice treated with clofibrate, a hypolipidemic drug and peroxisome proliferator, with the effect being greater for the 70 kDa component. The localization of the 68 kDa protein was shown to be completely integral to the peroxisome membrane. Although some 70 kDa protein was integral to the membrane, a significant proportion was released from the membrane by some procedures believed to detach peripheral proteins. The 70 kDa protein was also particularly susceptible to degradation during isolation – in particular, addition of EDTA to media used for isolation of peroxisomes resulted in membranes in which this protein was degraded to smaller immunoreactive fragments. These data have been discussed in relation to the significant clarification which they have provided of the status and characteristics of the major protein components of peroxisomal membranes.

Introduction

The membrane of mouse liver peroxisomes contains two high-molecular-mass integral proteins with subunit molecular masses of 68 and 70 kDa as the major constituents [1,2]. Treatment of mice with a number of structurally unrelated peroxisome proliferators results in a substantial increase in the proportion of the 70 kDa integral membrane protein (PMP 70) in peroxisome membranes, but with little or no change to the proportion of the 68 kDa integral membrane protein (PMP 68), or of other minor proteins [2].

A number of workers have studied the integral membrane protein composition of peroxisomes from rat liver [3–5] and seemingly conflicting reports have arisen as to the protein species which constitute this fraction. In particular, a number of the reported integral proteins have been reported to demonstrate immunochemical cross-reactivity [4,5], data which may indicate that fragmentation of higher-molecular-mass proteins has occurred during peroxisome isolation.

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To obtain meaningful data on the protein nature of the peroxisomal membrane, in vivo, we have felt it necessary to re-examine the characteristics of the major integral proteins. We have therefore prepared specific antisera against PMP 68 and PMP 70 isolated from mouse liver peroxisomal membranes and have undertaken an immunochemical analysis of these proteins in livers from normal and clofibrate-treated mice.

Materials and Methods

Materials

Mature female mice (Quackenbush strain) were obtained from the Central Animal Breeding House, Moggill, Brisbane. Clofibrate was a gift from I.C.I. Australia. *S. aureus* V8 proteinase was from Miles Scientific (Naperville, IL, U.S.A.). Protein molecular mass markers for SDS polyacrylamide gel electrophoresis were obtained from Pharmacia. ^{125}I for iodination, and autoradiography film (Hyperfilm-MP) were from Amersham. All other chemicals were of reagent grade.

Methods

Treatment of animals. Mature female mice weighing about 40 g had access to normal laboratory chow pellets and water ad libitum. For administration of clofibrate, chow pellets containing 0.35% (w/w) clofibrate were substituted for normal pellets for a period of 10 days.

Isolation of subcellular fractions. Highly purified peroxisomal and microsomal fractions were isolated from mouse liver, as previously described [1], but using the modifications described in Ref. [2]. Livers were excised from mice, finely minced using a tissue chopper, and homogenized in 7.5 ml of cold 0.25 M sucrose/0.1% ethanol using a Potter-Elvehjem homogenizer with a loose-fitting pestle. The pestle was rotated at 1000 rpm through one up-and-down passage. The homogenates were centrifuged at $2800 \times g$ for 15 min at 4°C in a Sorvall SS34 rotor, and the resultant pellets were suspended in sucrose/ethanol for biochemical analyses. The supernatants obtained were centrifuged at $16000 \times g$ for 10 min and the pellets obtained were washed in sucrose/ethanol and recentrifuged to obtain light mitochondrial fractions. This procedure represents a slight modification of a procedure previously reported by us, in which 1 mM glutaraldehyde was also included to stabilize peroxisomal membranes [1].

The light mitochondrial pellets were gently resuspended in sucrose/ethanol and centrifuged through metrizamide gradients to resolve the peroxisomes, as previously described [1,2]. The post-light mitochondrial supernatants were centrifuged at $27000 \times g$ for 15 min to pellet residual large organelles, and finally centrifuged at $100000 \times g$ for 60 min (Beckman 50Ti rotor) to obtain a microsomal pellet. The medium used for homogenization was 0.25 M sucrose/0.1% ethanol, except for the experiments where different homogenization media were compared. These variations in the normal procedure are indicated in the text. The purity of the microsomal fraction was 97%, on the basis of NADPH-cytochrome *c* reductase activity, and peroxisomal contamination was 0.5% (catalase and urate oxidase as markers). The data relating to peroxisome purity are presented in Table I.

Mitochondria were isolated from livers of mice which had been injected with Triton WR-1339, using modifications of the method of Leighton et al. [6]. The modifications involved using a heavy mitochondrial fraction prepared from livers as described by Bustamante et al. [7], but with 0.25 M sucrose/0.1% ethanol as the homogenizing medium. This fraction was suspended to a volume of 0.7 ml in the homogenizing medium then loaded onto a 12 ml sucrose gradient containing dextran T10 [6] and centrifuged at 40000 rpm ($180000 \times g_{av}$) for 90 min in a Beckman SW 41 rotor on a L8-55 Beckman ultracentrifuge. The gradient was subsequently fractionated and the peak mitochondrial fraction was identified by marker enzyme analysis. Mitochondrial purity, on the basis of cytochrome oxidase activity, was 93%. Contamination by peroxisomes, as judged by activity of catalase and urate oxidase, was 0.5%.

Membranes were isolated from the purified organelles using the carbonate procedure of Fujiki et al. [8], and then solubilized in SDS-sample buffer for protein analysis and SDS-polyacrylamide gel electrophoresis.

Isolation of PMP 68 and PMP 70. These two major peroxisomal integral membrane proteins were first resolved by SDS-polyacrylamide gel

electrophoresis (7% separating gel) of peroxisomal integral membrane fractions, using the precautions recommended by Hunkapiller et al. [9]. After electrophoresis, the proteins were visualized by incubating the gel in 4 M sodium acetate for 20 min [10]. The bands corresponding to the two major proteins were cut out, rinsed briefly with water, and processed immediately for electroelution. The electroelution was based on the 'cold-room procedure' of Hunkapiller et al. [9] *, but using the apparatus described by Lazarides [11]. After electroelution, the samples were lyophilized and the protein was precipitated at −30°C with 90% ethanol, and finally dissolved in 0.9% sodium chloride. The isolated proteins were homogeneous and had unchanged subunit molecular masses when analysed by SDS-polyacrylamide gel electrophoresis (data not shown).

Preparation of antisera. Antisera against PMP 68 and PMP 70 were prepared by inoculation of the isolated proteins into both popliteal lymph nodes of rabbits (10 μg protein per node), according to the method described by Sigel et al. [12], except that the nodes were located without the need for skin incisions. Booster injections (20 μg protein) were given subcutaneously along the back until the antibody titres were satisfactory.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [13], but as modified for slab gels by Ames [14]. 7–15% gradient gels were normally used for polypeptide separation and immunoblotting, except for the peptide mapping experiments where 10–15% gradient gels were used. Gels were stained either with Coomassie brilliant blue R [2], or by the silver procedure of Morrissey [15].

Immunoblotting. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose was performed according to the method of Towbin et al. [16], but using the carbonate blot buffer described by Dunn [17]. Following transfer, nitrocellulose membranes were incubated briefly in phosphate-buffered saline containing 1% bovine serum albumin to block protein sites. Antiserum was added to this solution (1/2000 dilution), and the membranes shaken overnight at room temperature. The membranes were then washed extensively in phosphate-buffered saline/0.05% Triton X-100, and then in phosphate-buffered saline alone. The washed nitrocellulose membranes were incubated for 3 h at 37°C with phosphate-buffered saline/bovine serum albumin containing ^{125}I-labelled protein A (approx. $2 \cdot 10^5$ cpm/ml), washed again as above, and the dried membrane was exposed to autoradiography film at −70°C for 1 or 2 days.

Analytical methods. Catalase, cytochrome *c* oxidase, β-glucuronidase and urate oxidase were assayed, as previously described [1]. Protein was determined according to Peterson [18]. Protein in membrane samples which had been dissolved in SDS-sample buffer was determined in the same manner, after prior heating of samples in a boiling water bath for 10 min to remove interference by 2-mercaptoethanol [19].

Iodination of protein molecular mass markers. The protein molecular mass markers were iodinated according to the method described by Hunter and Greenwood [20].

Partial peptide mapping. Proteins were separated by 7% SDS-polyacrylamide gel electrophoresis. The proteins for mapping were excised from the gels and the slices were loaded into the wells of a 10–15% gradient SDS-polyacrylamide gel and digested at 12°C with varying amounts of *S. aureus* V8 proteinase, as described by Cleveland [21]. The digest products were then separated by electrophoresis and identified by silver staining or immunoblotting.

Results

Isolated peroxisomes

The peroxisomal fractions used in these experiments were assessed for purity through estimation of organelle marker enzyme activities as described previously [1], using the distribution of organelle protein content reported by Leighton et al. [6] for rat liver. As shown in Table I, the peroxisomal fractions purified from livers of control mice and from clofibrate-treated mice contained 90.5 ± 2.7% and 96.5 ± 0.6% peroxisomal protein, respectively.

* Although PMP 68 was able to be recovered intact by electroelution at room temperature, PMP 70 was shown to be almost completely degraded when subsequently analysed by electrophoresis.

TABLE I

MARKER ENZYME ACTIVITIES IN PEROXISOMAL FRACTIONS PURIFIED FROM MOUSE LIVER

Data represent mean ± S.D. for three determinations. Catalase, cytochrome oxidase and NADPH-cytochrome *c* reductase are markers for peroxisomes, mitochondria and endoplasmic reticulum, respectively.

	Marker enzyme	Specific activity			% Organelle [c] contribution
		homogenate [a]	peroxisome [a]	relative [b]	
Control mice	catalase	335 ±51	12150 ±1760	36.3 ±1.1	90.5±2.7
	cytochrome oxidase	0.38 ± 0.19	0.020 ± 0.007	0.071±0.060	1.4±1.2
	NADPH-cytochrome *c* reductase	0.035± 0.002	0.0076± 0.0007	0.21 ±0.02	4.5±0.4
Clofibrate-treated mice	catalase	606 ±73	7030 ± 250	11.8 ±1.9	96.5±0.6
	cytochrome oxidase	0.47 ± 0.14	0.0028± 0.0010	0.007±0.005	0.1±0.1
	NADPH-cytochrome *c* reductase	0.046± 0.004	0.0072± 0.0018	0.16 ±0.03	3.4±0.6

[a] Specific activity is in U/mg protein. Enzyme units (U) are: catalase, μmol H_2O_2/min; cytochrome oxidase, μmol cytochrome *c* oxidized/min; NADPH-cytochrome *c* reductase, μmol cytochrome *c* reduced/min.

[b] Relative specific activity is the ratio of the specific activity in the peroxisomal fraction to the specific activity in the homogenate.

[c] % Organelle contribution is calculated from the relative specific activity data, as in Ref. 1, assuming the contribution of organellar protein in mouse liver is the same as that described by Leighton et al. [6] for rat liver.

Lack of homology between PMP 68 and PMP 70

PMP 68 and PMP 70 from membranes of purified peroxisomes were compared using two unrelated analyses. In the first analysis, the proteins were subjected to limited proteolysis by *S. aureus* V8 proteinase in SDS-polyacrylamide gels [21] in order to obtain partial peptide maps. The results, shown in Fig. 1, demonstrated that the partial peptide maps of PMP 68 and PMP 70 were different. Immunoblotting techniques were employed as the second method for comparing the properties of PMP 68 and PMP 70. As shown in Fig. 2, the antisera raised against PMP 68 reacted with PMP 68, as expected, but did not cross-react with PMP 70. Similarly, antisera against PMP 70 did not cross-react with PMP 68.

Subcellular distribution of PMP 68 and PMP 70

The distribution of PMP 68 and PMP 70 in the major organelles was tested by means of immunoblotting with antisera against PMP 68 and PMP 70, respectively (Fig. 3). It was found that, in peroxisome membranes, only the proteins with the expected molecular masses were recognized by the antisera; in the total peroxisomal fractions, as well as the expected high-molecular-mass species a number of lower-molecular-mass polypeptides

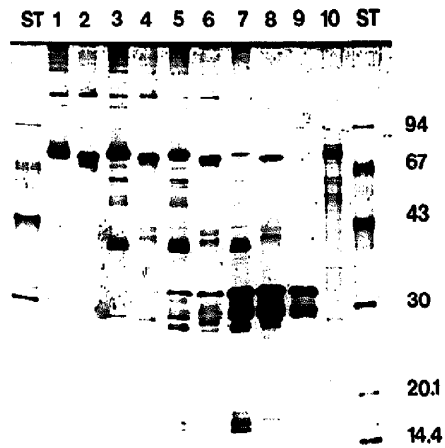

Fig. 1. Peptide mapping of PMP 68 and PMP 70 by limited proteolysis and analysis by SDS-polyacrylamide electrophoresis. PMP 68 and PMP 70 were separated and digested with V8 proteinase as described in the Methods section. The digest products were identified by silver staining. Lanes 1, 3, 5, 7, PMP 70; lanes 2, 4, 6, 8, PMP 68. V8 proteinase was loaded into lanes 3 to 9: lanes 3 and 4, 100 ng; lanes 5 and 6, 300 ng; lanes 7 and 8, 1 μg; lane 9, 1 μg of proteinase alone. Lane 10, peroxisomal membrane proteins. ST, molecular mass standards: phosphorylase *b* (94 kDa), bovine albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa).

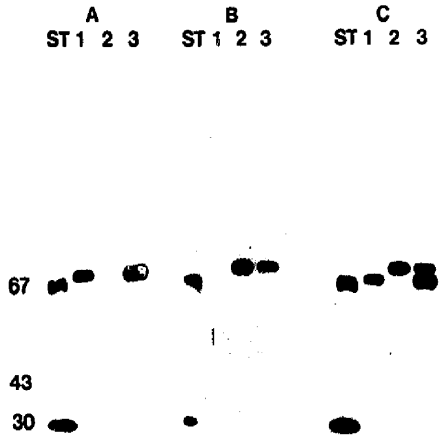

Fig. 2. Immunoblot analysis of PMP 68 and PMP 70. Total peroxisomal integral membrane proteins and gel pieces containing either PMP 68 or PMP 70 were loaded into the wells of a 7% SDS-polyacrylamide gel and the proteins were separated by electrophoresis, and immunoblotted using antisera raised against each of the proteins. Immunoblot with (A) anti-PMP 68; (B) anti-PMP 70; (C) anti-PMP 68 and anti-PMP 70. Lane 1, PMP 68; lane 2, PMP 70; lane 3, peroxisomal membrane. ST, molecular mass standards; the standards used for immunoblots were radioactively labelled with Iodine-125.

were identified by the antisera, these presumably representing proteolytic degradation products of the native proteins which are able to be extracted by carbonate treatments (see below). None of the proteins in the purified mitochondrial or microsomal fractions, or in the the membranes from these fractions, reacted with the antisera.

The distribution of PMP 68 and PMP 70 in the membrane fractions and soluble fractions of both liver homogenates and purified peroxisomal fractions was also investigated (Fig. 4). The results indicated that PMP 68 was found exclusively in the integral membrane fractions resulting from carbonate extraction (Fig. 4B). PMP 70 was also observed in the membrane fractions, as expected; however, the carbonate-soluble fractions from both homogenates and purified peroxisomes also contained a 70 kDa polypeptide species which was recognized by the anti-PMP 70 sera (Fig. 4C). This distribution of anti-PMP 70-immunoreactive protein between the carbonate membrane and carbonate-soluble fractions from mouse liver was unchanged by the addition of proteinase inhibitors (10 μg/ml leupeptin/1 mM phenylmethylsulphonyl fluoride) to the homogenizing media, or by directly homogenizing liver in cold carbonate solution (data not shown). It is of interest that a minor polypeptide with molecular mass of approx. 35 kDa was also identified in the homogenate-soluble fractions, by antisera against PMP 70. These experiments also served to demonstrate that the amounts of PMP 68 and PMP 70 were enhanced in livers of mice treated with clofibrate, with the effect being about 3-fold greater for PMP 70 than for PMP 68 (Fig. 4B,C).

In order to investigate the relationship between PMP 70 and the 70 kDa polypeptide detected in the carbonate soluble fractions, partial peptide mapping in combination with immunoblotting was employed. The peptide maps recognized by the antisera, of PMP 70 from peroxisomal membranes and the 70 kDa protein from peroxisomal carbonate-soluble fractions, were identical (data not shown).

The effect of various homogenizing media used in the preparation of peroxisomes on the composition of peroxisomal integral membrane proteins

The general medium used for liver homogenization in the preparation of peroxisomal fractions was 0.25 M sucrose/0.1% ethanol. We investigated the effect on the peroxisomal integral membrane protein profile of modifications to this homogenizing medium. The results are shown in Fig. 5. Of particular note was that peroxisomes isolated in a medium containing 10 mM glycylglycine (pH 7.5) exhibited an approx. 3-fold increase in the proportion of PMP 70 in the integral membrane fractions, when compared to in control preparations, whereas when this medium contained 1 mM EDTA as well, the proportion of PMP 70 was reduced to less than 10% of the level seen in the absence of EDTA. The effect of EDTA addition appeared to be general, regardless of the medium used, in that not only was the amount of PMP 70 reduced, but there was increased amounts of polypeptides with lower molecular masses. This is most clearly seen in the preparations where 50 mM potassium phosphate buffer (pH 8)/1 mM EDTA was used. These lower-molecular-mass polypeptides were recognized by the anti-PMP 70 sera

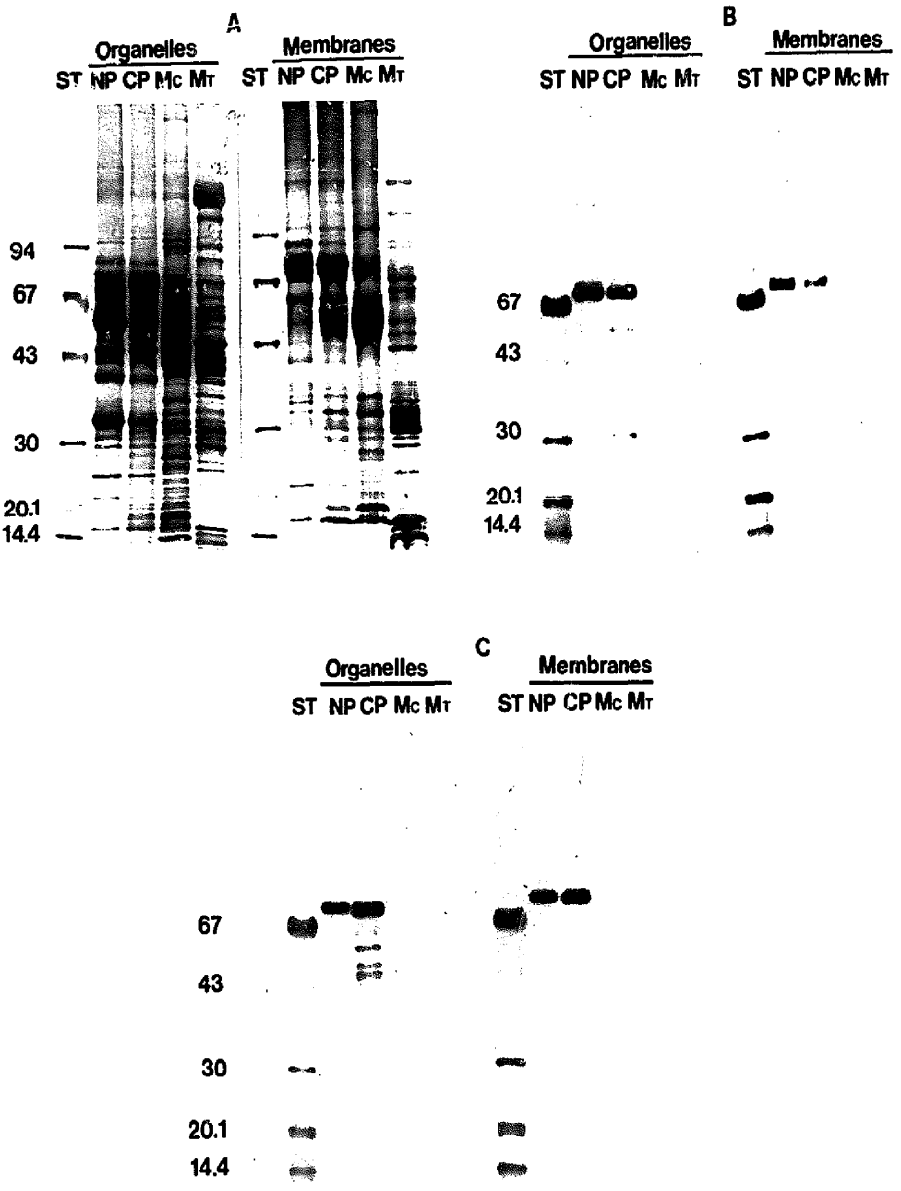

Fig. 3. Specificity of anti-PMP 68 and anti-PMP 70 antisera, as evaluated by immunoblot analysis of purified peroxisomal, mitochondrial and microsomal fractions, and membranes prepared from these fractions by carbonate extraction. (A) Coomassie brilliant blue protein stain; (B) Immunoblot with anti-PMP 68; (C) Immunoblot with anti-PMP 70. For the protein stain, 50 μg of protein from organelles and membranes were analysed; for immunoblots, analysis was of 25 μg of organellar protein and 5 μg of membrane protein. NP, peroxisomes from normal mouse liver; CP, peroxisomes from livers of clofibrate-treated mice; Mc, total microsomes; Mt, mitochondria; ST, molecular mass standards.

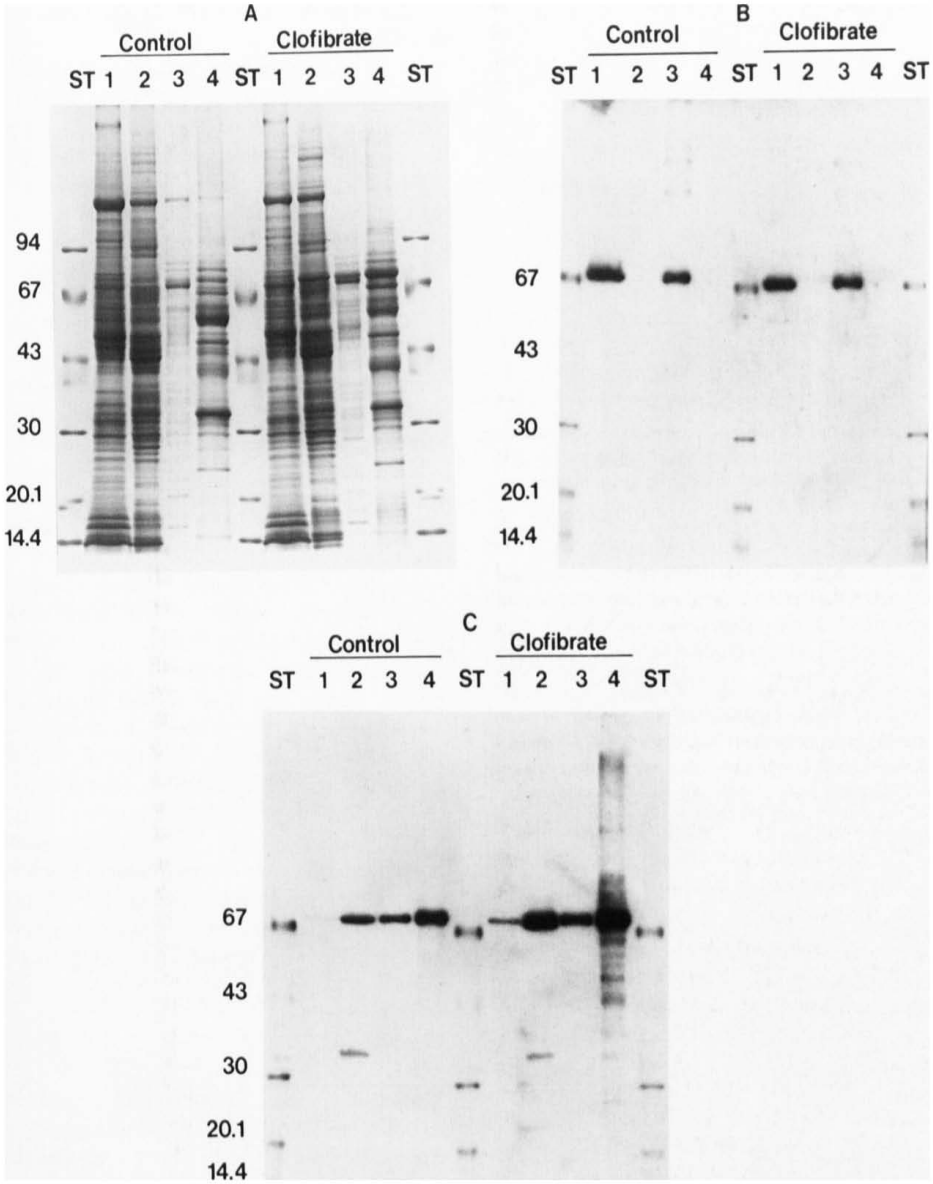

Fig. 4. Immunoblot analysis of the distribution of PMP 68 and PMP 70 following carbonate extraction of liver homogenates and purified peroxisomal fractions from control and clofibrate-treated mice. (A) Coomassie brilliant blue protein stain; (B) Immunoblot with anti-PMP 68; (C) Immunoblot with anti-PMP 70. Lanes 1 and 2, membranes and soluble proteins, respectively, prepared by carbonate extraction of liver homogenates; lanes 3 and 4, membranes and soluble proteins, respectively, prepared by carbonate extraction of peroxisomes. ST, molecular mass standards. For the protein stain, analysis was made of 75 μg homogenate membrane protein, 75 μg homogenate soluble protein, 10 μg peroxisomal membrane protein and 50 μg peroxisomal soluble protein. For the immunoblots, analysis was of 200 μg protein from homogenate fractions from control mice, of 75 μg protein from clofibrate-treated mice, of 5 μg peroxisomal membrane protein and 25 μg peroxisomal soluble protein from both control and clofibrate-treated mice.

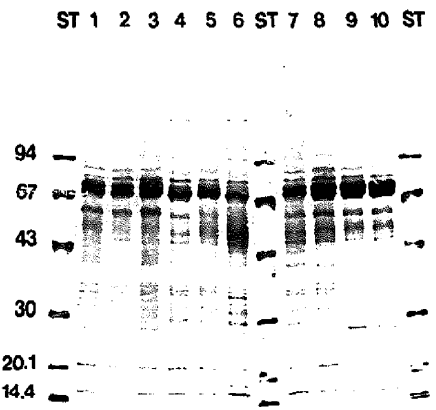

Fig. 5. Integral membrane protein composition of peroxisomes prepared using different media for liver homogenization. Peroxisomes were purified and 15 μg of integral membrane protein fraction, prepared by carbonate extraction, were analysed by electrophoresis in a 7–15% gradient SDS-polyacrylamide gel and stained with Coomassie brilliant blue. All homogenizing media contained 0.25 M sucrose/0.1% ethanol. Other additions to this media were as follows: lane 1, no addition; lane 2, 0.1 mM leupeptin; lane 3, 10 mM glycylglycine buffer (pH 7.5); lane 4, 10 mM glycylglycine buffer/1 mM EDTA (pH 7.2); lane 5, 10 mM glycylglycine buffer/1 mM EDTA (pH 7.5); lane 6, 50 mM potassium phosphate buffer/1 mM EDTA (pH 8); lanes 7 and 9, no addition; lanes 8 and 10, 10 mM glycylglycine buffer (pH 7.2). Lanes 1 to 8 are preparations from control mice; 9 and 10 are from clofibrate-treated mice. ST, molecular mass standards. All peroxisome preparations were more than 90% pure on the basis of organelle marker-enzyme activities.

(data not shown). In addition, immunoblotting experiments demonstrated that PMP 68 was also substantially degraded in membranes when EDTA was used, giving rise to two breakdown products at 42 and 28 kDa (data not shown). The effects of the different media were similar when using livers from control mice or from clofibrate-treated mice.

Solubilization of PMP 70

Various procedures known to be able to detach peripheral membrane proteins [8] were employed in order to gauge the degree of association of PMP 70 with the membrane (data not shown). Extraction with sodium carbonate at 100 mM, our usual method, released more than half of the protein from peroxisomal membranes. Repeated extractions of this type did not result in any further solubilization. Extraction with 90 mM sodium hydroxide resulted in the loss of both PMP 70 and PMP 68 from the membrane and an increase in degradation products of PMP 70. *p*-Chloromercuribenzoate solubilized only a small proportion of PMP 70, while treatment with 5 mM EDTA or 0.05% sodium deoxycholate resulted in no solubilization of this component at all.

Sequential extraction of PMP 70 from peroxisomes by increasing concentrations of Triton X-100

When peroxisomes were treated with Triton X-100, the amount of PMP 70 which was solubilized increased as the concentration of the detergent was increased (Fig. 6). It was, however, substantially more difficult to extract PMP 70 than it was the components of the peroxisomal matrix, such as catalase, or the components of peroxisome core, such as urate oxidase. As shown in lane 3 of Fig. 6, when most of catalase and about 50% of urate oxidase in peroxisomes had been extracted, only a small proportion of the PMP 70 was solublized. When 85 mM Na_2CO_3 was used, catalase and urate oxidase were almost completely extracted, whereas almost all of PMP 70 was still membrane-bound (Fig. 6, lane 6). By comparison, 100 mM Na_2CO_3 was able to extract all the catalase and urate oxidase, as well as more than 50% of PMP 70, from peroxisomal fractions (Fig. 4A and C). Findings similar to those with Triton X-100 extraction were obtained from experiments directed at the sequential extraction of peroxisomal components, using increasing concentrations of sodium deoxycholate up to 0.4% (data not shown).

Discussion

Since the recent description of satisfactory methods for the isolation of peroxisomes from mammalian liver, there has been a considerable effort to characterize the polypeptide components which constitute the integral membrane protein fraction in this organelle [1–5]. Using carbonate extraction of peroxisomes to obtain an integral membrane protein fraction [3], for example, a number of research groups have identified proteins with subunit molecular masses of 68 and 70 kDa representing two of the major components of

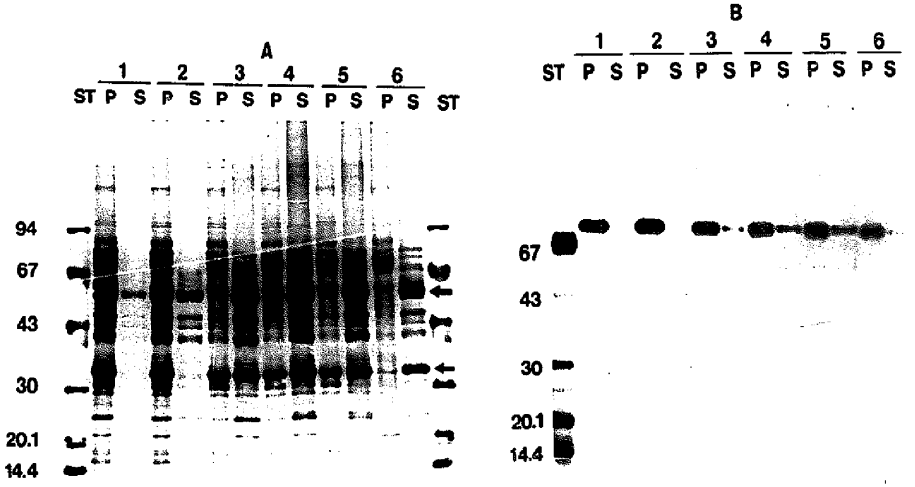

Fig. 6. Sequential extraction of PMP 70 from peroxisomes by increasing concentrations of Triton X-100. Peroxisomes purified from livers of control mice were incubated for 30 min on ice with various concentrations of Triton X-100 and then centrifuged at 100000×*g* for 1 h to produce a pellet (P) and a supernatant (S) fraction. (A) Coomassie brilliant blue stain of proteins separated by 7–15% SDS-polyacrylamide gel electrophoresis; (B) Immunoblot with anti-PMP 70 of proteins separated as in (A); Triton X-100 concentrations, expressed as mg Triton X-100/mg peroxisomal membrane protein prepared by carbonate extraction, in lanes 1. 2, 3, 4, and 5, were 0, 10, 25, 50 and 100 mg Triton X-100/mg protein, respectively. Note that the highest concentration used is equivalent to 0.2% (w/v). Fractions in lane 6 are those resulting from extraction with Na_2CO_3 (85 mM).

this fraction [1,3,4]. Hashimoto et al. [4] have suggested that the 68 kDa protein is derived from the 70 kDa protein via leupeptin-sensitive proteolysis. Our data, however, which comprise comparisons of partial peptide mapping, immunological cross-reactivity and stability characteristics, indicate that the two polypeptides are structurally unrelated, although a partial homology between the proteins cannot be excluded at this stage.

The data we have presented here in this report also reflect significantly on the question as to what form the membrane protein profile takes in vivo, and particularly the use of isolated peroxisomal fractions to assess this condition. We have demonstrated that the method of preparation of peroxisomal fractions has a profound influence on the profile of integral membrane proteins which is subsequently observed. In particular, the content of PMP 70 in this fraction varies depending on the isolation method, and seems to be particularly sensitive to the presence of EDTA in isolation buffers. In fact, use of the buffer system described by Just and co-workers [5,22], in our hands, results in almost complete degradation of PMP 70 to lower-molecular-mass polypeptides, some of which remain in the membrane fraction. On the other hand, incubation of a peroxisomal fraction (prepared under our standard conditions) with 5 mM EDTA (pH 7.5), resulted in no loss of PMP 70. These results suggest that the effect of EDTA during isolation procedures is manifested via an activation of proteolytic enzymes, to which PMP 70 is susceptible. PMP 68 is also susceptible to degradation when peroxisomes are prepared in EDTA containing solutions, although not to the extent seen with PMP 70. These latter experiments, interestingly, identified breakdown products of PMP 68 at 42 and 28 kDa, and have led us to conclude that our PMP 68 is probably equivalent to the PMP 69 described by Hartl and Just [5], although the induction of the latter protein by clofibrate does not reconcile with our previous work that identifies PMP 70, and not PMP 68, as the component which is increased in membranes by hypolipidemic drugs [2].

In these experiments we have also examined the degree of association of PMP 68 and PMP 70 with the peroxisome membrane. Under all conditions

tested, PMP 68 remained associated with the integral membrane fraction, and we thus conclude that it represents a tightly membrane-associated integral membrane protein. The situation with PMP 70 is more complex, in that the use of different extraction procedures demonstrated that, although some of the protein remained associated with the membrane, a significant portion was able to be solubilized – a result which was particularly evident in the case of extraction with 100 mM sodium carbonate. However, less solubilization of this protein occurred with other procedures which still appeared to extract peroxisomal content and core proteins as well as peripheral membrane proteins. Experiments employing extraction with 85 mM sodium carbonate, or with increasing concentrations of Triton X-100 or sodium deoxycholate, were able to illustrate this particular aspect. These results indicate a relatively tight association of some PMP 70 with the peroxisomal membrane, but the fact that only a portion of this protein displays the membrane-binding properties characteristics of PMP 68 points to some heterogeneity with respect to membrane association for this component. These data have also raised the question as to the identity of PMP 70 – in particular, the inducibility and solubility characteristics show some similarities to those reported for the bifunctional protein (enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase) of peroxisomes [3,23]. Experiments are at present underway to test this possibility.

In summary, the data from the present studies are considered to provide a valuable amplification and clarification of the available information on the characteristics and interrelationships of the major proteins in the peroxisomal membrane. As such, the data provide a useful basis for further experimentation into peroxisomal function. In particular, the successful isolation of PMP 68 and PMP 70, and the preparation of monospecific antisera to these proteins, will allow examination of the genetic regulation of these peroxisomal-membrane components, experiments which are now in progress.

Acknowledgement

The receipt of financial support from the National Health and Medical Research Council of Australia is gratefully acknowledged.

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