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Synthesis of a major integral membrane polypeptide of rat liver peroxisomes on free polysomes

(organelle biogenesis/sodium carbonate membrane isolation procedure/specific antibody/immunoblot/cell-free protein synthesis)

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ABSTRACT **The manner of synthesis and assembly of the peroxisomal membrane proteins is unknown. Understanding these processes is essential to an understanding of the formation of the organelle. We have investigated the biogenesis of the previously identified major 21.7-kDa integral peroxisomal membrane polypeptide [Fujiki, Y., Fowler, S., Shio, H., Hubbard, A. L. & Lazarow, P. B. (1982) *J. Cell Biol.* 93, 103–110]. This protein was purified to apparent homogeneity and used to elicit a rabbit antiserum. In immunoblotting analysis, antibody bound only to the 22-kDa membrane polypeptide present exclusively in peroxisomal membranes. Total rat liver RNA was translated in a nuclease-treated rabbit reticulocyte cell-free protein-synthesizing system. The *in vitro* translation product, isolated by means of the antibody and *Staphylococcus aureus* cells, comigrated with the mature 22-kDa polypeptide in NaDodSO$_4$/PAGE. Analysis of the translation products of RNAs from free and membrane-bound polysomes indicated that the mRNA for the 22-kDa membrane polypeptide is found predominantly in free polysomes. The results imply post-translational insertion of the membrane polypeptide into the peroxisomal membrane without proteolytic processing and suggest that peroxisomes, like mitochondria and chloroplasts, form by fission from preexisting organelles.**

How intracellular organelles are formed is of great interest. Studies of the biogenesis of peroxisomes have focused on matrix enzymes such as catalase (the most abundant protein of rat liver peroxisomes) (1–8), the enzymes catalyzing peroxisomal fatty acid β-oxidation (9–11, 47), and the crystalloid core proteins, urate oxidase (rat liver) (4) and alcohol oxidase (methanol-grown yeast) (12). These "content" proteins are synthesized on free polysomes and enter peroxisomes post-translationally, in most cases without proteolytic processing.

Knowledge of where and how the membranes of peroxisomes are formed is essential to understanding the biogenesis of these organelles. Several mutually exclusive mechanisms for the biogenesis of peroxisomal membrane proteins have been considered (13). Integral membrane proteins (IMPs) could be incorporated directly into peroxisomal membranes post-translationally, after synthesis on free polysomes of the cytosol, in a fashion similar to the packaging of content proteins. Alternatively, membrane proteins could be synthesized on membrane-bound polysomes, cotranslationally inserted into rough endoplasmic reticulum membranes, and subsequently sorted into the peroxisomal membranes via transient connections between endoplasmic reticulum and peroxisomal membranes, as early morphological evidence suggested (14). Direct cotranslational insertion by polysomes bound to peroxisomal membranes represents a third possibility, which, however, is not supported by any morphological evidence.

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Previously, we isolated peroxisomal and endoplasmic reticulum membranes as flat sheets by means of a sodium carbonate procedure that removed peripheral membrane proteins (15). We found that the IMPs of these two endomembranes are almost entirely different, but that their phospholipid compositions are similar (16). Among peroxisomal IMPs, a 21.7-kDa polypeptide was a major species that was apparently unique to peroxisomes.

As a step toward understanding the synthesis and assembly of the peroxisomal membrane, we have studied the biogenesis of this 22-kDa IMP. We report its isolation from rat liver, the preparation of a specific rabbit antibody against it, its synthesis *in vitro*, and the subcellular site of its mRNA. Some of these results have appeared in abstract form (17).

MATERIALS AND METHODS

Isolation of the 22-kDa IMP of Peroxisomes. Peroxisomal membranes were prepared by sodium carbonate treatment of highly purified rat liver peroxisomes (15, 16, 18) and subjected to preparative NaDodSO$_4$/PAGE in 1.8-mm-thick 10–15% acrylamide slab gels (18 × 31 cm). After guide strips from both sides of the gel were stained with Coomassie brilliant blue, the region of the unstained gel containing the 22-kDa IMP was cut out. The polypeptide was eluted electrophoretically from the gel slices in 50 mM Tris/glycine buffer, pH 8.3/0.1% NaDodSO$_4$ and lyophilized after dialysis against 50 mM NH_4HCO_3.

Preparation of Antisera. Thirty micrograms of the 22-kDa IMP was suspended in 150 μl of distilled water, emulsified with an equal volume of complete Freund's adjuvant, and injected into the popliteal lymph nodes of both hind legs of an anesthetized rabbit (19). Eleven weeks later, 25 μg of the 22-kDa IMP was emulsified in incomplete Freund's adjuvant and injected subcutaneously into two sites, and 5 μg of protein suspended in 200 μl of saline was injected into an ear vein. The rabbit was bled two weeks later and thereafter at weekly intervals.

Rabbit anti-rat liver catalase was described previously (20) and rabbit anti-rat serum albumin was purchased from United States Biochemical (Cleveland, OH).

Isolation and Translation of RNA. RNA was isolated from the livers of male Fisher F-344 rats. Total RNA was prepared by guanidinium thiocyanate/guanidinium hydrochloride extraction (21). Free and membrane-bound polysomes were isolated by the modified procedure (22) of Ramsey and Steele (23). RNA was isolated from these polysomes by the guanidinium thiocyanate procedure of Ullrich *et al.* (24) as modified by Raymond and Shore (25). Equal amounts of free and membrane-bound polysomal RNAs (760 μg/g of liver) were obtained by this procedure, in agreement with previous results (22). The messenger activity of free polysomal RNA (47,000 cpm/μg of RNA) was about twice that of membrane-bound polysomal RNA (25,000 cpm/μg of RNA). The isola-

Abbreviation: IMP, integral membrane protein.

tion of free and membrane-bound RNAs from the liver of a clofibrate-treated rat was described elsewhere (10, 47).

RNAs were heated at 65°C for 5 min and cooled in ice water to improve translational efficiency (26, 48). They were then translated at a final concentration of 200–600 μg/ml at 26°C for 60 min, using [^{35}S]methionine and a micrococcal nuclease-treated rabbit reticulocyte lysate translating system (27).

Immunoprecipitation of Cell-Free Product. Translation mixtures were diluted 10-fold and adjusted to 1% Nonidet P-40/10 mM Tris Cl, pH 7.4/150 mM NaCl/10 mM methionine/leupeptin (25 μg/ml)/antipain (25 μg/ml)/chymostatin (12.5 μg/ml)/pepstatin (12.5 μg/ml). The diluted translation mixtures were centrifuged for 1 hr at 40,000 rpm in a Beckman type 50 Ti rotor (maximum, 150,000 × *g*) at 4°C to yield post-ribosomal supernatants. $NaDodSO_4$ and EDTA were added to final concentrations of 0.1% and 2 mM, respectively. The post-ribosomal supernatants were then incubated with 13 μl of rabbit antiserum or preimmune serum for 90 min at room temperature and then overnight at 4°C. Antigen–antibody complexes were adsorbed for 90 min at room temperature onto inactivated *Staphylococcus aureus* cells [63 μl of a 10% suspension that had been prewashed with binding buffer (10 mM Tris Cl, pH 7.4/1% Nonidet P-40/0.1% $NaDodSO_4$/150 mM NaCl/10 mM methionine/2 mM EDTA containing the same concentrations of protease inhibitors as given above)]. The *S. aureus* cells were collected by centrifugation and washed sequentially in a Microfuge tube with binding buffer (three washes), 10 mM Tris Cl, pH 7.4/150 mM NaCl/0.05% Nonidet P-40/10 mM methionine (two washes), 10 mM Tris Cl, pH 7.4/150 mM NaCl/10 mM methionine, and 10 mM Tris Cl, pH 7.4/10 mM methionine. All washing solutions contained the protease inhibitors. The immunoprecipitates were dissociated by boiling for 5 min in 50 μl of $NaDodSO_4$/PAGE sample buffer. After *S. aureus* cells were removed by centrifugation, the supernatants were analyzed by $NaDodSO_4$/PAGE in 7–15% acrylamide slab gels (15, 28) followed by fluorography using preexposed films (29, 30).

Preproalbumin and catalase cell-free translation products were similarly immunoselected by means of specific rabbit antibodies and were analyzed in the same fashion. Radioactive bands were quantitated by densitometric scanning of the fluorograms with a Bio-Rad Model 1650 densitometer.

Trypsin Treatment. Highly purified frozen peroxisomes (88 μg of protein) from normal rat livers were treated with 44–880 ng of trypsin (1:2000 to 1:100 trypsin/peroxisome ratios, wt/wt) in 50 μl of 100 mM Tris Cl, pH 8.5, at 0°C for 5 min. One half of the reaction mixture was then boiled in $NaDodSO_4$/PAGE sample buffer. The other half was subjected to the Na_2CO_3 procedure (15) to separate membranes from soluble proteins. These fractions were analyzed by $NaDodSO_4$/PAGE.

Other Methods. Amino acid analysis was performed with a Durrum D-500 amino acid analyzer after hydrolysis of protein with constant-boiling 5.7 N HCl at 110°C for 24 hr in a sealed evacuated tube. Immunoblotting was done by a modification of the procedure of Burnette (31), using 1% bovine hemoglobin instead of 5% bovine serum albumin. The antigen–antibody–[^{125}I]-labeled protein A complex was detected by autoradiography at −80°C on Kodak X-Omat AR-film with Cronex Lightning Plus intensifying screens (Dupont). Protein was determined by the Lowry method (32) with bovine serum albumin as a standard. Total microsomal and purified mitochondrial fractions were prepared from rat liver as described (15, 16).

Animals and Materials. Rats were purchased from Charles River Breeding Laboratories. The rabbit was from Dutchland (Denver, PA). [^{35}S]Methionine (specific activity, 1000–1300 Ci/mmol, 1 Ci = 37 GBq) was purchased from Amersham. The following were from Sigma: bovine hemoglobin type II, protease inhibitors (antipain, chymostatin, leupeptin, and pepstatin), and molecular mass marker proteins [rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), bovine pancreas trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa), bovine milk β-lactoglobulin (18.4 kDa), egg white lysozyme (14.3 kDa), and bovine lung trypsin inhibitor (6.5 kDa)]. Formaldehyde-fixed, heat-inactivated *S. aureus* (Pansorbin) was purchased from Calbiochem–Behring. Complete and incomplete Freund's adjuvants were obtained from Difco. ^{125}I-labeled protein A was purchased from New England Nuclear. X-Omat AR films were from Eastman. All other chemicals were of analytical grade.

RESULTS

Purified 22-kDa Peroxisomal IMP. The 22-kDa IMP, purified by cell fractionation, membrane isolation, preparative $NaDodSO_4$/PAGE, and electroelution, migrated as a single homogeneous band when analyzed by $NaDodSO_4$/PAGE (Fig. 1). Amino acid analysis revealed that 44% of the amino acids of the protein are hydrophobic (Table 1).

Trypsin Sensitivity. Mild trypsinization of isolated peroxisomes at 0°C caused the disappearance of the 22-kDa IMP (Fig. 2, arrows) with the concomitant appearance of a slightly smaller polypeptide of ≈21 kDa (triangles). The smaller polypeptide was observed in total peroxisomes (lanes P) and in membranes isolated by the carbonate procedure (15) (lanes M), indicating that it is still integral to the membrane. The peroxisomes had been frozen and thawed prior to the trypsinization; nonetheless, although repeated freezing and thawing gradually releases peroxisomal proteins (33), electron microscopy shows that the peroxisomal membrane appears to be (re)sealed even after 10 cycles of freezing and thawing (unpublished observations). The smallest amount of trypsin that effectively attacked the 22-kDa IMP (1:1000 ratio of trypsin to total peroxisomal protein) also degraded the 68- and 70-kDa membrane polypeptides described previously (16) (Fig. 2, lanes M) but did not much affect the content proteins (lanes S). This suggests that the 22-kDa IMP may be exposed on the cytosolic side of the peroxisomal membrane.

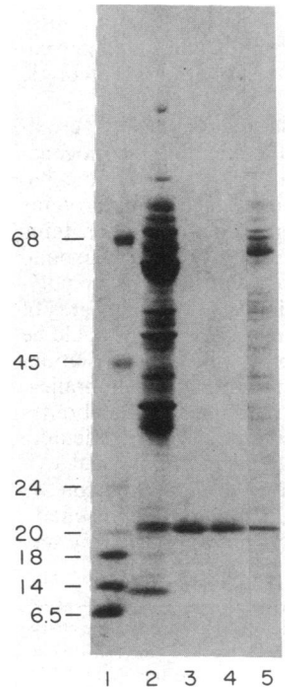

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoretic analysis of the isolated 22-kDa IMP of rat liver peroxisomes. Lanes: 1, molecular mass marker proteins [molecular masses (in kDa) are given to the left]; 2, purified rat liver peroxisomes (50 μg); 3 and 4, two different preparations of the 22-kDa IMP; 5, peroxisomal membranes prepared from 100 μg of peroxisomes by the sodium carbonate procedure.

Table 1. Amino acid composition of the 22-kDa IMP

Amino acid	Residues/molecule	Mol %
Asx	15	8.0
Thr	8	4.3
Ser	13	7.0
Glx	18	9.6
Pro	9	4.8
Gly	18	9.6
Ala	16	8.6
Cys*	1	0.5
Val	12	6.4
Met	6	3.2
Ile	8	4.3
Leu	22	11.8
Tyr	8	4.3
Phe	9	4.8
His	3	1.6
Lys	11	5.9
Arg	10	5.3
Trp	ND	—
Total	187	100.0%

Amino acid residues were calculated on the basis of His = 3. Values are rounded to the nearest integer. A molecular mass of 20,538 was calculated for the protein, exclusive of tryptophan. ND, not determined.
*Determined as *S*-carboxymethylcysteine.

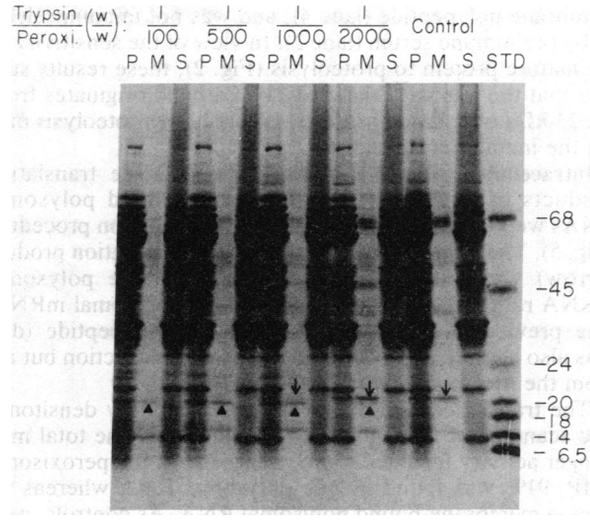

FIG. 2. Trypsin treatment of peroxisomes at 0°C for 5 min. Lanes: P, peroxisomes (total reaction mixture); M, membranes prepared by the Na_2CO_3 procedure; S, soluble proteins. The weight ratios of trypsin to peroxisomal protein (Peroxi.) are given at the top of each set of lanes. Control, no trypsin added. STD, molecular mass marker proteins (values in kDa at right). The arrows and triangles indicate the 22-kDa IMP and an ≈21-kDa polypeptide, respectively.

Immunoblots. Rabbit antiserum raised against the purified 22-kDa IMP was used as the probe in an immunoblot analysis of liver fractions (Fig. 3). Antibodies bound to the 22-kDa IMP in total rat liver peroxisomes (Fig. 3*B*, lane 5) and in membranes prepared therefrom by the carbonate procedure (lane 6). Antibodies did not bind to any other peroxisomal protein (lanes 5 and 6) nor to any polypeptide in either mitochondrial or microsomal fractions (lanes 2, 3, 8, and 9). The antibody also bound to only the 22-kDa IMP in liver peroxisomes and peroxisomal membranes of rats treated with clofibrate, a hypolipidemic drug that causes extensive proliferation of hepatic peroxisomes (35) as well as striking increases in peroxisomal enzyme activities (36) and proteins (34) (lanes 4 and 7). These results show that the antiserum is specific and that the 22-kDa polypeptide is located solely in peroxisomes.

Cell-Free Protein Synthesis. Immunoselection among the rabbit reticulocyte cell-free translation products of total rat liver RNA with anti-22-kDa IMP gave a major ^{35}S-labeled band of 22 kDa on $NaDodSO_4$/PAGE (Fig. 4, lane 3). This [^{35}S]polypeptide was indistinguishable in electrophoretic mobility from mature authentic 22-kDa IMP (lane 1, arrow), was displaced during immunoprecipitation by unlabeled authentic 22-kDa IMP (lane 4), and was not precipitated by preimmune serum (lane 2). A minor [^{35}S]polypeptide with slightly lower molecular mass (≈21 kDa) was also immunoselected (lane 3, dot), was displaced by unlabeled authentic

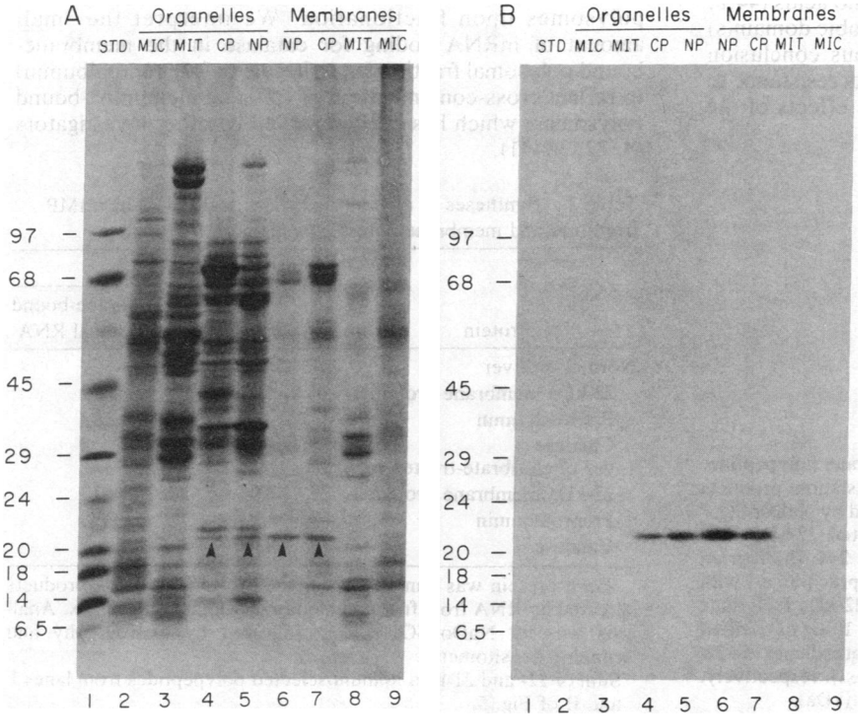

FIG. 3. Immunoblot analysis. (*A*) Gel stained with Coomassie brilliant blue. (*B*) Immunoblot with rabbit anti-22-kDa IMP and ^{125}I-labeled protein A. Lanes: 1, molecular mass markers (STD, values in kDa given to left); 2, total microsomes of rat liver (MIC), 50 μg; 3, mitochondria (MIT), 50 μg; 4, peroxisomes (30 μg) purified from liver of rats treated with clofibrate (CP) (34); 5, peroxisomes (50 μg) from normal rat liver (NP); 6, membranes from 100 μg of normal peroxisomes, prepared by the Na_2CO_3 procedure; 7, membranes from 100 μg of clofibrate-induced peroxisomes; 8, membranes from 100 μg of mitochondria; 9, membranes from 100 μg of total microsomal fraction. Arrowheads in *A* indicate the 22-kDa IMP.

membrane polypeptide (lane 4), and was not immunoselected by preimmune serum (lane 2). In view of the sensitivity of the mature protein to proteolysis (Fig. 2), these results suggest that the minor ^{35}S-labeled 21-kDa band originates from the 22-kDa translation product, probably by proteolysis during the immunoprecipitation.

Intracellular Site of Synthesis. The cell-free translation products of total, free, and membrane-bound polysomal RNAs were subjected to the immunoprecipitation procedure (Fig. 5). The immunoprecipitated 22-kDa translation product (arrow) was encoded predominantly by free polysomal mRNA rather than by membrane-bound polysomal mRNA. The previously observed ≈21 kDa [^{35}S]polypeptide (dot) was also abundantly translated from the free fraction but not from the membrane-bound fraction.

The translation products were quantitated by densitometric scanning of the fluorogram (Table 2). Of the total messenger activity for the cell-free synthesis of the peroxisomal IMP, 91% was found in free polysomal RNA whereas 9% was in membrane-bound polysomal RNA. As controls, catalase and preproalbumin translation products were immunoprecipitated and analyzed by $NaDodSO_4$/PAGE, fluorography, and densitometry. As shown in Table 2, [^{35}S]catalase was 92% in the free polysomal RNA translation products whereas [^{35}S]preproalbumin was 98% in the membrane-bound polysomal RNA translation products. These findings confirm previous reports that catalase is synthesized on free polysomes (4, 10, 47) and show that the separation of free and membrane-bound polysomes was adequate. Similar analysis of messenger activity from the liver of rats treated with clofibrate showed that the 22-kDa IMP is synthesized on free polysomes under these conditions as well (Table 2).

DISCUSSION

We have reported previously that the peroxisomal membrane in rat liver has a unique polypeptide composition (16). Prominent among the integral proteins in this membrane is one, with a mass of 21.7 kDa (22-kDa IMP), that could not be detected in other endomembranes by $NaDodSO_4$/PAGE and Coomassie-blue staining (16). The immunoblot analysis presented above confirms that the 22-kDa IMP is located solely in peroxisomal membranes.

The 22-kDa IMP is rich in hydrophobic amino acids (44%, see Table 1), which presumably form hydrophobic domain(s) embedded within the membrane. Our previous conclusion that this polypeptide is an IMP was based on its resistance to extraction by carbonate at pH 11.5 (16). The effects of the carbonate procedure were evaluated using microsomal fractions; carbonate treatment removed microsomal content proteins and peripheral membrane proteins as it converted microsomal vesicles to flat membrane sheets that retained IMPs and enzymes (15). Alkalinity (pH 12) is known to selectively remove peripheral membrane proteins from erythrocyte membranes (37). The finding that the 22-kDa IMP contains 44% hydrophobic amino acids is consistent with the conclusion that it is an IMP. Mild proteolysis (Fig. 2) suggests that the 22-kDa IMP may be exposed on the cytosolic face of the peroxisomal membrane; whether it is also exposed on the internal face is unknown.

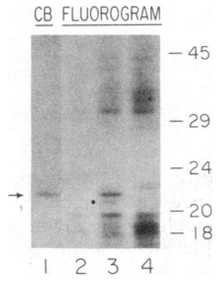

FIG. 4. *In vitro* synthesis of the 22-kDa membrane polypeptide. Immunoselected [^{35}S]polypeptides from the translation products (130 μl, 1.24×10^6 cpm) of total RNA were analyzed by $NaDodSO_4$/PAGE and fluorography. Lane 1, 2 μg of isolated 22-kDa IMP stained with Coomassie brilliant blue (CB). Lanes 2–4, fluorogram showing the labeled translation products immunoprecipitated with preimmune serum (lane 2), with antiserum to the 22-kDa IMP (lane 3), and with the same antiserum in the presence of 10 μg of purified unlabeled authentic 22-kDa IMP. The arrow and dot indicate the 22-kDa IMP and the ≈21-kDa [^{35}S]polypeptide (see text), respectively. Numbers to the right are molecular mass markers (kDa).

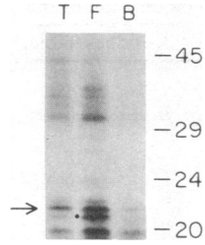

FIG. 5. Site of synthesis of the 22-kDa IMP. Fluorogram of immunoselected cell-free products from translation of total RNA (T), free polysomal RNA (F), and membrane-bound polysomal RNA (B). Molecular mass markers, in kDa, are to the right. The same amount of radioactivity (1.24×10^6 cpm) from each translation mixture was subjected to immunoprecipitation. The arrow and dot indicate the 22-kDa and the 21-kDa [^{35}S]polypeptides, respectively.

Our investigation of the biogenesis of the 22-kDa IMP was designed to distinguish between two principal alternatives: (*i*) budding of the peroxisomal membrane from endoplasmic reticulum, which implies cotranslational insertion of peroxisomal integral membrane proteins into endoplasmic reticulum, followed by sorting; (*ii*) post-translational assembly of newly made membrane proteins into preexisting peroxisomes. The finding that the mRNA for the 22-kDa IMP is predominantly in free polysomes (both in normal and in clofibrate-treated rats) fits the second but not the first alternative. This finding also excludes the possibility of synthesis by polysomes bound to peroxisomal membranes, which presumably would accompany endoplasmic reticulum-bound polysomes upon fractionation. We interpret the small amount of mRNA coding for catalase in the membrane-bound polysomal fraction (and vice versa for preproalbumin) to reflect cross-contamination of free and membrane-bound polysomes, which has been observed by other investigators (4, 22, 38–41).

Table 2. Syntheses of albumin, catalase, and the 22-kDa IMP from free and membrane-bound polysomal RNA

Protein	% of total	
	Free polysomal RNA	Membrane-bound polysomal RNA
Normal rat liver		
22-kDa membrane protein*	91	9
Preproalbumin	2	98
Catalase	92	8
Liver of clofibrate-treated rat		
22-kDa membrane protein	83	17
Preproalbumin	2	98
Catalase	93	7

Each protein was immunoprecipitated from translation products directed by RNA from free and membrane-bound polysomes. Analysis was by $NaDodSO_4$/PAGE, followed by fluorography and scanning densitometry.

*Sum of 22- and 21-kDa immunoselected polypeptides from lanes F and B of Fig. 5.

These results may be interpreted in the light of our knowledge of the biogenesis of other organelles. So far as is presently known, the nuclear gene-encoded proteins of intracellular organelles are either all made on free polysomes (mitochondria and chloroplasts) or all made on membrane-bound polysomes (lysosomes) (42). Our observation that a major peroxisomal IMP is made on free polysomes (like all peroxisomal content proteins studied so far) suggests that peroxisomes fall into the same category as mitochondria and chloroplasts—i.e., that they form by fission from preexisting peroxisomes, as suggested previously (13). On the other hand, we also know that, although most endoplasmic reticulum proteins are synthesized by membrane-bound polysomes, two are made on free polysomes (22, 39, 40). This raises uncertainty about the generality of the results reported here. Therefore, no firm conclusion can be drawn concerning the formation of the peroxisomal membrane until the biogenesis of other peroxisomal IMPs is examined.

The 22-kDa IMP is synthesized *in vitro* as such, rather than as a larger precursor. In this respect, it resembles most peroxisomal content proteins investigated, including matrix enzymes such as catalase (4, 5, 7, 8), fatty acyl-CoA oxidase and hydratase-dehydrogenase (two enzymes belonging to the peroxisomal β-oxidation system; refs. 9, 10, 47), carnitine octanoyltransferase (11), and urate oxidase (the crystalloid core protein; ref. 4). These findings imply post-translational peroxisomal uptake of newly synthesized polypeptides without proteolytic processing. Indeed, fatty acyl-CoA oxidase and catalase are so imported post-translationally into peroxisomes *in vitro* (43).

Evidently, the information directing the 22-kDa IMP to the peroxisomal membrane is inherent in its structure. In this respect, it resembles a minority of mitochondrial IMPs that also are synthesized at their mature sizes [e.g., ADP/ATP carrier protein (44), porin (45), and monoamine oxidase (46)]. The mechanism of insertion of newly synthesized 22-kDa IMP into the peroxisomal membrane remains to be determined.

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