Acyl-CoA Oxidase and Hydratase-Dehydrogenase, Two Enzymes of the Peroxisomal β-Oxidation System, Are Synthesized on Free Polysomes of Clofibrate-treated Rat Liver

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ABSTRACT   We investigated the site of synthesis of two abundant proteins in clofibrate-induced rat hepatic peroxisomes. RNA was extracted from free and membrane-bound polysomes, heated to improve translational efficiency, and translated in the mRNA-dependent, reticulocyte-lysate–cell-free, protein-synthesizing system. The peroxisomal acyl-CoA oxidase and enoyl-CoA hydratase–β-hydroxyacyl-CoA dehydrogenase ^{35}S-translation products were isolated immunochemically, analyzed by SDS PAGE and fluorography, and quantitated by densitometric scanning. The RNAs coding for these two peroxisomal proteins were found predominantly on free polysomes, and the translation products co-migrated with the mature proteins. As in normal rat liver, preproalbumin and catalase were synthesized mainly by membrane-bound and by free polysomes, respectively. mRNAs for a number of minor ^{35}S-translation products also retained by the anti-peroxisomal immunoadsorbent were similarly found on free polysomes. These results, together with previous data, allow the generalization that the content proteins of rat liver peroxisomes are synthesized on free polysomes, and the data imply a posttranslational packaging mechanism for these major content proteins.

The peroxisome is a subcellular organelle, bounded by a unit membrane, that in liver functions in respiration, lipid metabolism, purine and polyamine catabolism, and other metabolic pathways (1, 2). The biogenesis of the peroxisome has been the subject of controversy. On the basis of electron microscopic and biochemical studies of rat liver, it was long believed that the peroxisome forms by budding from the endoplasmic reticulum (3–12). However, studies of the synthesis of two rat liver peroxisomal proteins suggested otherwise (13–19). Catalase, the principal matrix protein of normal rat liver peroxisomes (13), is synthesized at its final size on free polysomes, and enters peroxisomes in vivo posttranslationally as an apomonomer without detectable proteolytic (or other) processing (14–17). Urate oxidase, located in the paracrystalline peroxisomal core, is also synthesized on free polysomes at its final size (15, 19).

We therefore decided to investigate the synthesis of other rat liver peroxisomal proteins to determine whether synthesis on free polysomes is a generality for peroxisomal proteins or whether catalase and urate oxidase are exceptions to the rule, just as cytochrome b_5 (20) and cytochrome b_5 reductase (21) are exceptions to the rule that endoplasmic reticulum proteins are made on bound polysomes (22).

In these studies, we made use of clofibrate, a hypolipidemic drug that causes peroxisome proliferation (23) and strongly induces the peroxisomal β-oxidation system (24). This system consists of three proteins: an acyl-CoA oxidase (AOx),[1] a bifunctional enoyl-CoA hydratase–β-hydroxy acyl-CoA dehydrogenase (HD), and thiolase (25). HD becomes the most abundant protein in peroxisomes of clofibrate-treated rats and AOx increases to probably fifth in abundance, as determined by Coomassie Blue staining (26, Mortensen et al., in preparation). We investigated the site of synthesis of HD and AOx, using mRNA-dependent reticulocyte lysate to translate RNA from either free or membrane-bound polysomes of rat liver

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[1] *Abbreviations used in this paper:* AOx, acyl-CoA oxidase; HD, hydratase-dehydrogenase.

treated with clofibrate. The results have been communicated in abstract form (27).

MATERIALS AND METHODS

A Fisher F-344 male rat was treated for 7 d with 0.5% (wt/wt) clofibrate in Purina rat chow (Ralston Purina Co., St. Louis, MO). Free and membrane-bound polysomes were isolated by the procedure of Ramsey and Steele (28) as modified by Rachubinski et al. (20). RNA was isolated from these polysome populations by the guanidinium thiocyanate procedure of Ullrich et al. (29) as modified by Raymond and Shore (30). Total RNA was isolated from a second clofibrate-treated rat liver by guanidinium thiocyanate/guanidinium hydrochloride extraction (31). All RNAs were routinely dissolved in sterile distilled deionized (Millipore system, Millipore Corp., Medford, MA) H_2O. Aliquots

TABLE I

Yields of RNAs from Liver of Clofibrate-treated Rats and Activities of RNAs in Cell-free Translation

	Yield	Activity
	μg RNA/g liver	cpm × 10³/μg RNA*
Total RNA	3,400	42
Free polysomal RNA	860	45
Membrane-bound polysomal RNA	900	19

* Hot-trichloroacetic acid-precipitable radioactivity. All RNAs were heated at 65°C for 5 min followed by rapid cooling on ice-water, immediately before translation.

were heated at 65°C for 5 min in microfuge tubes, and then rapidly cooled in ice-H_2O immediately before translation (32). Cell-free translation was performed for 1 h as described (33, 34) in an RNA-dependent, nuclease-treated rabbit reticulocyte lysate system with [³⁵S]methionine (1 mCi/ml, 1,000–1,300 Ci/mmol) as the labeled amino acid. After translation, lysates were centrifuged at 150,000 g_{max} for 1 h in the Beckman 50 Ti rotor (Beckman Instruments, Inc., Fullerton, CA) at 4°C to remove ribosomes and polysomes. The pattern of ³⁵S-polypeptides seen by SDS PAGE and fluorography was not affected by this centrifugation.

Peroxisomal cell-free translation products were isolated by immunoaffinity chromatography with a polyclonal goat antiserum. A goat was immunized with 22 mg of highly purified normal rat liver peroxisomes prepared according to Leighton et al. (35); serum was collected, beginning 1 mo later. A globulin fraction prepared by 30% $(NH_4)_2SO_4$ precipitation was coupled to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) at a final concentration of 25 mg of protein/ml of settled beads (36). Protein samples to be immunoadsorbed (~0.5–10 μl of translation mixture) were incubated with 0.5 ml of PBS (50 mM sodium phosphate, pH 7.4, 0.15 M NaCl) containing 0.5% (wt/vol) deoxycholate, 0.5% (vol/vol) Triton X-100 and 5 mM methionine, and 100 μl of solid-phase immunoadsorbent for 30 min at 4°C with constant mixing in a microfuge tube. The gel material was then transferred to a column (0.8 × 4 cm) and washed with 4 ml of 0.15 M borate, pH 8.6, containing 0.1% (wt/wt) Triton X-100, 1 M NaCl and 5 mM methionine, and then with 5 ml of 5 mM ammonium bicarbonate, pH 8.3, containing 100 U/ml apoprotinin and 5 mM methionine (procedure of L. Wong, Louisiana State University Medical Center, New Orleans, unpublished observations). Material bound to the gel was eluted with 300 μl of 15 mM Tris-Cl, pH 6.8, containing 0.5% (wt/vol) SDS and 1 mM dithiothreitol by incubating/mixing at 4°C for 30 min. Any remaining elution buffer was washed from the column with 300 μl of distilled H_2O. The eluant was concentrated on a Savant Speed Vac Concentrator (Savant Instru-
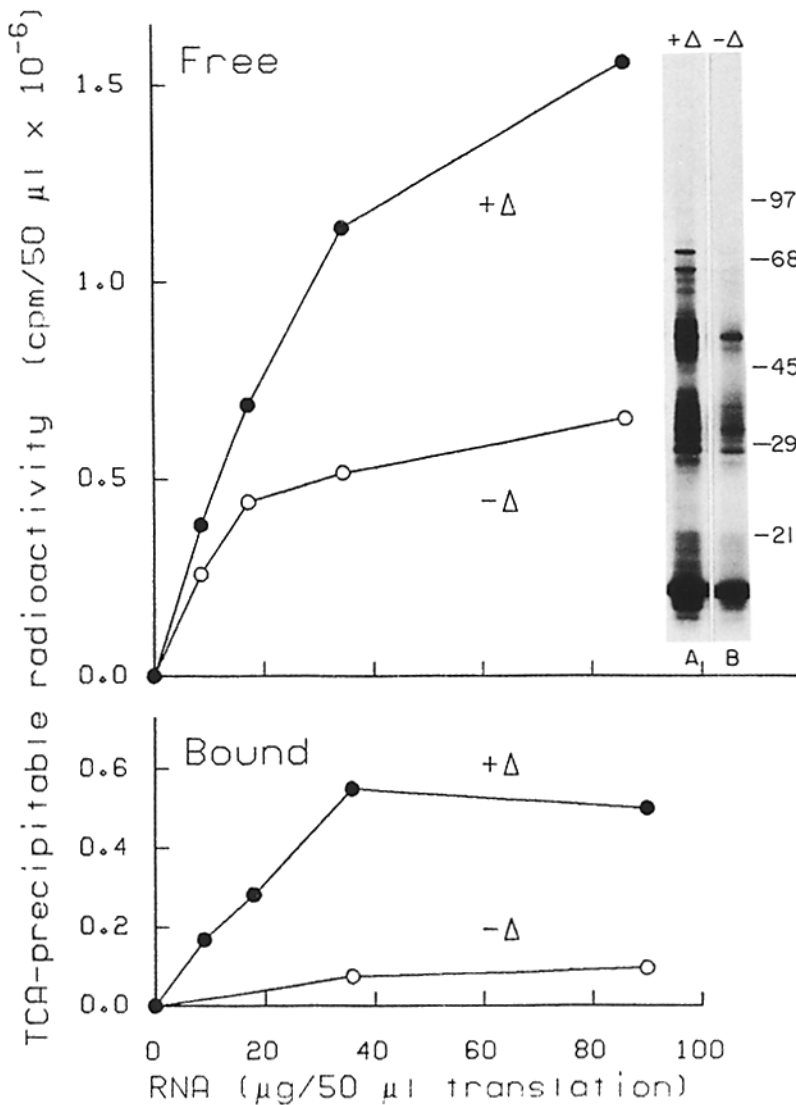

FIGURE 1 In vitro translation of free (upper panel) and membrane-bound (lower panel) polysomal RNA preparations from clofibrate-treated rat liver, demonstrating the effect of pretranslational heating on total hot trichloroacetic acid-precipitable radioactivity. (●) RNA heated at 65°C for 5 min and rapidly cooled, immediately before in vitro translation. (○) Not heated. Endogenous incorporation of [³⁵S]methionine into trichloroacetic acid-precipitable products without added RNA has been subtracted. (Inset) SDS PAGE fluorographic analysis of the translation products synthesized from free polysomal RNA (0.3 μl translation mixture). (Lane A) Heated (+Δ). (Lane B) Not heated (−Δ). Numbers on the right indicate the masses of molecular markers (in kdaltons): rabbit muscle phosphorylase b (97); bovine serum albumin (68); ovalbumin (45); bovine erythrocyte carbonic anhydrase (29); soybean trypsin inhibitor (21); and egg white lysozyme (14). Bovine serum albumin migrates somewhat slowly for its size in our experience. Fluorogram exposed 12 d.

ments, Inc., Hicksville, NY) and brought up to 37.5 μl with distilled H₂O and processed for SDS PAGE (37) and fluorography (38).

To immunoprecipitate in vitro-synthesized catalase, we diluted the translation mixture 10 times to final concentrations of 1% (wt/vol) Nonidet P-40, 10 mM Tris-Cl (pH 7.4), 0.15 M NaCl, 20 mM methionine, and 0.02% (wt/vol) sodium azide. The diluted translation mixture was centrifuged for 1 h at 150,000 g_{max}, and the supernatant was adjusted to 1.15 M NaCl and 0.9 mM phenylmethylsulfonyl fluoride. 270 μl of supernatant was incubated with 50 μl of rabbit anticatalase antiserum (39) for 90 min (with rotation) at room temperature and overnight at 4°C. Protein A (100 μl of a 10% suspension of inactivated *Staphylococcus aureus* cells (Pansorbin, Calbiochem-Behringer Corp., La Jolla, CA) was then added, and incubated at room temperature with rotation for 2 h. The *S. aureus* cells were pelleted in a microfuge and washed sequentially with (*a*) 0.15 M NaCl buffered with 10 mM sodium phosphate, pH 7.4 (PBS), containing 1% (wt/vol) Nonidet P-40 and 20 mM methionine; (*b*) 0.1% (wt/vol) SDS; (*c*) PBS; and (*d*) 0.15 M NaCl. The immune complexes were dissociated from the cells by boiling in SDS sample buffer and analyzed by SDS PAGE and fluorography as described above.

Highly purified peroxisomal bifunctional protein (HD) and acyl-CoA oxidase (AOx) were as described previously (26).

RESULTS

The procedure for isolating free and membrane-bound polysomal RNAs yielded approximately equal amounts of these two RNAs from clofibrate-treated rat liver (Table I). RNA from both free and membrane-bound polysomes stimulated protein synthesis in the rabbit reticulocyte lysate system (Fig. 1). Heating of RNA before in vitro translation increased template activity substantially, and the linearity of the translation system was extended to higher concentrations of RNA (Fig. 1). As shown previously for total guanidinium thiocyanate-extracted RNA (32), the heating does not affect the translation of all mRNAs equally. Rather, the increase in translation of certain mRNAs coding for large polypeptides was much more pronounced, especially in the region from M_r 60,000 to 80,000, where several peroxisomal polypeptides of interest are located (Fig. 1, inset). The template activities of the heated RNA preparations, estimated from the linear portions of the curves (Fig. 1), are summarized in Table I. The activity of the free polysomal RNA was 2.4 times that of the membrane-bound polysomal RNA. The activity of the total RNA (which was extracted from a liver homogenate without the isolation of polysomes) was approximately equal to that of the free polysomal RNA. All subsequent translations were performed at RNA concentrations in the linear portion of the curve.

The polypeptides encoded by the three RNA preparations were analyzed by SDS PAGE and fluorography (Fig. 2). Membrane-bound and free polysomal RNAs coded for different populations of polypeptides. The synthesis of preproalbumin, a protein known to be synthesized exclusively by membrane-bound polysomes (40), was directed almost entirely by RNA from membrane-bound polysomes. A number of other polypeptides were synthesized predominantly by free polysomal RNA (Fig. 2). These data demonstrate the successful separation of free and membrane-bound polysomes from clofibrate-treated rat liver. The translation products of both these classes of RNA are present among the translation products of total RNA (Fig. 2, lane *1*).

Peroxisomal polypeptides were immunoselected from translation products of total RNA by immunoaffinity chromatography using goat antiperoxisome antibodies (Fig. 2, lane 4). The two highly labeled [³⁵S]polypeptides immunoselected under these conditions were identified as acyl-CoA oxidase (AOx) and the bifunctional hydratase-dehydrogenase (HD) by competition with the purified enzymes (Fig. 3). At least 12 smaller translation products were retained by the

antiperoxisome immunoadsorbent (Fig. 2, lane *4*). Many of these are probably peroxisomal polypeptides, but we do not exclude the possibility that some are nonspecifically adsorbed.

The synthesis of both HD and AOx was directed preferentially by RNA from free polysomes (Fig. 2, compare lanes 5 and 6). The other 12 polypeptides retained by the antiperoxisome antibody column were also preferentially synthesized by free polysomal RNA.

The in vitro synthesis of catalase was investigated by using monospecific rabbit anticatalase (39). As shown in Fig. 4,

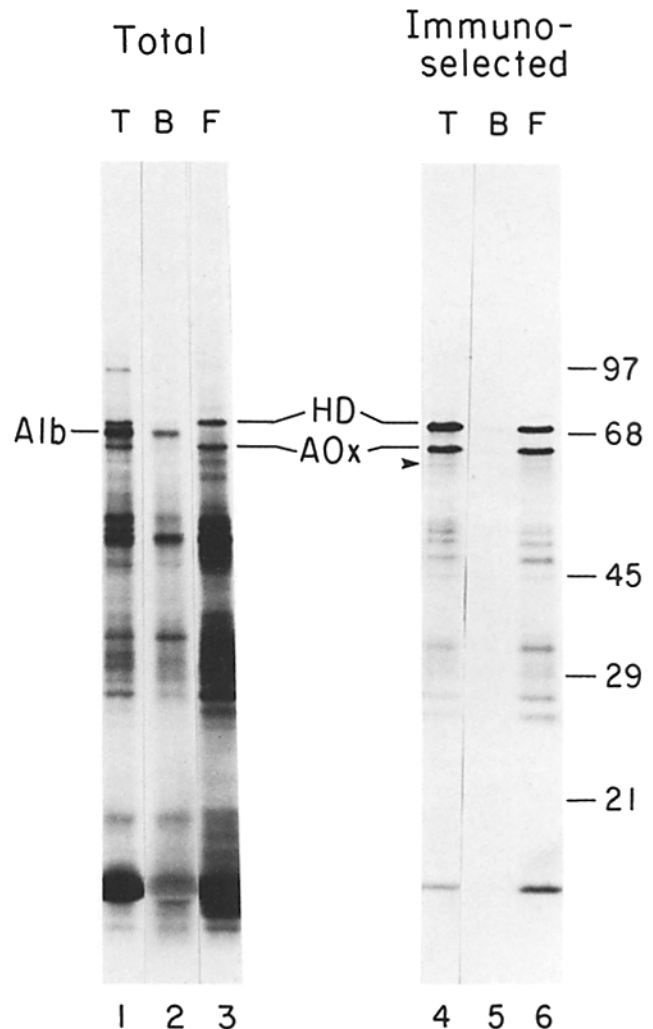

FIGURE 2 In vitro translation of RNA from total, membrane-bound, and free polysomes; fluorogram of total translation products and of products immunoselected by means of antiperoxisome antibody chromatography. (Left) Total translation products of total liver RNA (lane *1*), of membrane-bound polysomal RNA (lane 2), and of free polysomal RNA (lane 3). Lanes 1–3 each contained 0.3 μl of translation mixture: lane *1* had 12 μg RNA/50 μl translation mixture; lane 2 was from pooled aliquots of the four translations shown in Fig. 1 (lower panel, solid circles); lane 3 was from pooled aliquots of the four translations shown in Fig. 1 (upper panel, solid circles). (Right) Immunoselected translation products of total liver RNA (lane 4), membrane-bound polysomal RNA (lane 5), and free polysomal RNA (lane 6). Immunoselection was carried out on 3 μl of translation mixtures as in lanes 1–3. (Alb) preproalbumin. (AOx) peroxisomal acyl-CoA oxidase. (HD) peroxisomal hydratase-dehydrogenase. (Arrowhead) Catalase. Numbers on the right correspond to masses of molecular markers (kdaltons). Fluorogram of the SDS polyacrylamide gel exposed 12 d.

catalase synthesis was directed by total and by free polysomal RNA (lanes *1* and *2*, respectively), but only poorly by bound polysomal RNA (lane *3*). No catalase synthesis occurred in the reticulocyte lysate cell-free protein-synthesizing system in the absence of exogenous RNA (lane *4*). The catalase translation product may also be seen as a faint band under AOx in Fig. 2, lanes *4* and *6* (arrowhead).

These results were quantitated by densitometric scanning of the fluorograms and computation of the areas under individual peaks by triangulation. As shown in Table II, template activity for the cell-free synthesis of HD, AOx, and catalase was localized in free polysomal RNA to the extent of 91%, 92%, and 83%, respectively. Our interpretation of these results is that the 8–17% of synthesis of these three proteins coded for by membrane-bound polysomal RNA most likely reflects the contamination of the bound polysomal fraction by a small
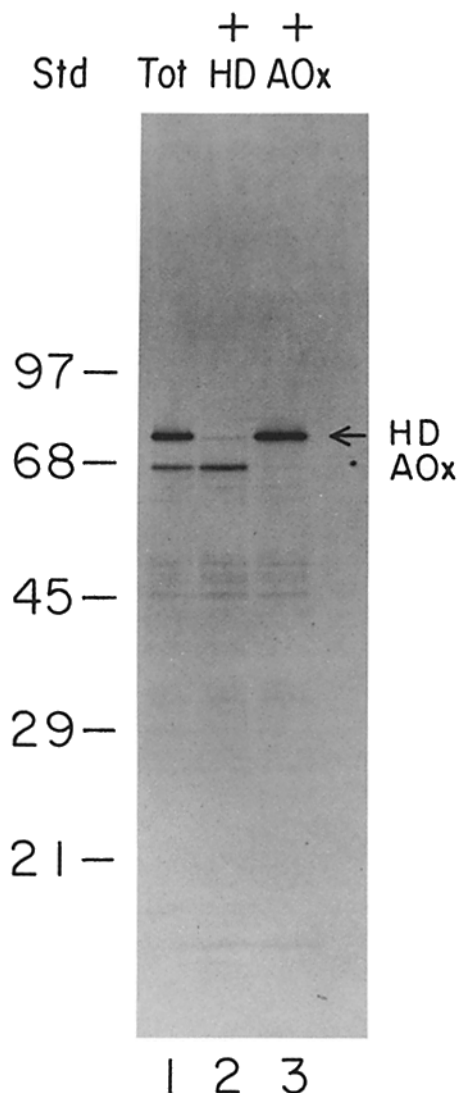

FIGURE 4   Synthesis of catalase. Catalase was immunoprecipitated from the translation products of total liver RNA (lane *1*), free polysomal RNA (lane *2*), membrane-bound polysomal RNA (lane *3*), and reticulocyte lysate without added exogenous RNA (lane *4*). Immunoprecipitation was done on 67,000 cpm of translation products directed by each RNA preparation. The dots (made with radioactive ink) indicate the position in the SDS polyacrylamide gel of the 66,000-mol-wt Coomassie Blue–stained subunit of catalase. The fluorogram was exposed for 1 mo in order to detect the band in lane *3*.

TABLE II

Quantitation of Immunoselected Products Synthesized in the Cell-free System by RNA from Free and Membrane-bound Polysomes*

Translation product	Free polysomal RNA		Membrane-bound polysomal RNA	
	A[‡]	B[§]	A[‡]	B[§]
	% of total			
Hydratase-dehydrogenase (HD)	91	92	9	8
Acyl-CoA oxidase (AOx)	93	91	7	9
Catalase		83		17
Preproalbumin[‖]		~100		

* Translation products directed by RNA from free or membrane-bound polysomes were submitted to immunoselection and SDS PAGE. The fluorograms were scanned with a Bio-Rad model 1650 densitometer and the areas under individual peaks were computed by triangulation. Synthesis by free (or bound) polysomal RNA is computed as a percentage of synthesis by free plus bound.
‡ Equal volumes of translation mixtures taken for immunoselection. Correction was made for the difference in total incorporation in cell-free synthesis. Fig. 2, lanes 5 and 6.
§ Equal numbers of total counts of translation products (after subtracting residual endogenous synthesis) taken for immunoselection. HD and AOx fluorogram not illustrated; catalase data from Fig. 4, lanes 2 and 3.
‖ Densitometry of Fig. 2, lanes 2 and 3. No preproalbumin peak could be discerned among the total translation products of free polysomal RNA. Presumably a small amount would be found if immunoprecipitation of albumin were carried out, as noted by other investigators (20, 21, 30, 41) and ourselves (47).

amount of free polysomes, as observed by other investigators (30, 20, 21, 41).

The sizes of the in vitro products were also investigated. The HD translation product co-migrated in SDS PAGE with mature HD of peroxisomes (Fig. 5). Similarly, the AOx translation product co-migrated with subunit A of AOx in peroxisomes (Fig. 5). The HD and AOx translation products also co-migrated with the corresponding purified enzymes (Fig. 3). In confirmation of previous observations (14, 15, 17), the catalase translation product co-migrated with the subunit of the mature enzyme (Figs. 4 and 5). We also observed co-migration of the HD, AOx, and catalase translation products with their mature counterparts when the SDS gel electrophoresis was carried out for 90 h instead of the usual 20 h (not illustrated). Thus, none of these peroxisomal proteins appears to be synthesized as a larger precursor. Furuta et al. (42) have reported that the HD and AOx in vitro translation products are approximately the same sizes as the mature proteins as judged by SDS PAGE.

+ +
Std Tot HD AOx

97 —

68 —   ← HD
       • AOx

45 —

29 —

2 I —

1 2 3

FIGURE 3   Identification of immunoselected cell-free translation products by competition with purified enzymes. (Lane *1*) Total products immunoselected with 40 μl of immunoadsorbent from 10 μl of translation mixture. (Lane *2*) Immunoadsorbent presaturated with 20 μg of hydratase-dehydrogenase (*HD*). (Lane *3*) Immunoadsorbent pre-saturated with 10 μg of acyl-CoA oxidase (*AOx*). The arrow and radioactive ink dot indicate the positions of the purified HD and AOx subunit A (25, 26, 48), respectively, in the SDS gel. Fluorogram exposed 2 d.
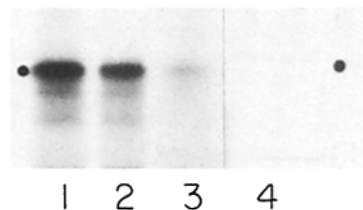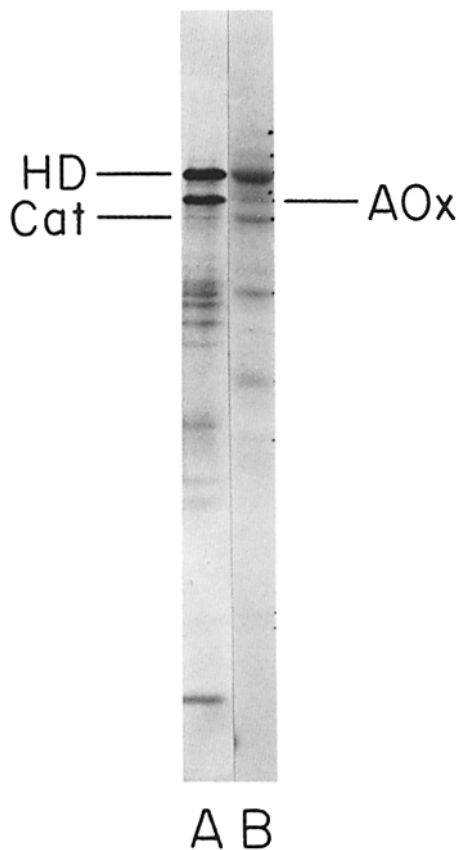
HD ———
Cat ——— ——— AOx

A B

FIGURE 5   Size comparison of peroxisomal polypeptides synthesized in vitro and in vivo. (Lane A) Fluorogram of immunoselected in vitro translation products. (Lane B) Coomassie Blue–stained SDS gel of clofibrate-induced peroxisomal polypeptides (15 µg) identified as described (13, 17, 26). Fluorogram exposed for 12 d.

DISCUSSION

These data, together with previous reports (13–15, 17, 26), permit us to draw two generalizations for the first time: the content proteins of rat liver peroxisomes are synthesized on free polysomes and (with the apparent exception of thiolase [42, 43]) they are made at their final sizes. These conclusions are based on the following facts. The two most abundant proteins in normal rat liver peroxisomes (catalase and urate oxidase), two of the most abundant proteins in clofibrate-treated rat liver peroxisomes (HD and AOx), and as many as a dozen other peroxisomal proteins are encoded by RNA located in free polysomes. Thus far, no peroxisomal protein has been found to be synthesized on membrane-bound polysomes. The four major proteins investigated, which include three matrix proteins and the main (if not only) constituent of the paracrystalline peroxisomal core (urate oxidase) are all synthesized in vitro at their final sizes, rather than as larger precursors.

These results are inconsistent with the old hypothesis that peroxisomal content proteins are synthesized by membrane-bound polysomes and then transported through endoplasmic reticulum cisternae to forming peroxisomes (3–12). The present data imply the posttranslational uptake of newly synthesized proteins into preexisting peroxisomes.

Elsewhere we report on investigations of the intracellular transport of peroxisomal proteins in vivo (44), the posttranslational uptake of acyl-CoA oxidase in vitro (45) and the

biogenesis of the major M_r 21,700 membrane protein (46, 47). These observations are fully consistent with the present findings and conclusions.

Methodologically, we have found that free and membrane-bound polysomes can be isolated from the liver of clofibrate-treated rats by the same procedure that is used for normal rat liver; the extent of cross-contamination of fractions is similar. As might be expected, clofibrate does not alter the site of synthesis of albumin or of catalase. The procedure of heating RNA before translation to increase translational efficiency (32) made it possible to carry out these experiments without months-long fluorographic exposures.

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