Proc. Natl. Acad. Sci. USA
Vol. 75, No. 10, pp. 5066–5070, October 1978
Cell Biology

Biogenesis of peroxisomes: Intracellular site of synthesis of catalase and uricase

(rat liver/free and membrane-bound polysomes/cell-free protein synthesis/immunoprecipitation with monospecific antisera against albumin, catalase, and uricase/polyacrylamide gel electrophoresis)

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Communicated by Alex B. Novikoff, August 3, 1978

ABSTRACT **The intracellular site of synthesis of two peroxisomal enzymes of rat liver, uricase (urate:oxygen oxidoreductase, EC 1.7.3.3) and catalase (hydrogen peroxide:hydrogen peroxide oxidoreductase, EC 1.11.1.6), has been localized on free ribosomes and not membrane-bound ribosomes. Free polysomes and membrane-bound polysomes, prepared by classical cell fractionation techniques from rat liver, were incubated for protein synthesis in a cell-free system derived from rabbit reticulocytes. Characterization of the total translation products by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, as well as by immunoprecipitation with anti-rat albumin antiserum, confirmed that good separation of the two polysome classes was achieved. Uricase and catalase were immunoprecipitable from translation products directed by free polysomes or phenol-extracted free polysomal mRNA but not from products of membrane-bound polysomes. Furthermore, unlike albumin, nascent uricase and catalase were not cotranslationally segregated by dog pancreas microsomal membranes. The results indicate that uricase and catalase are transferred to the interior of peroxisomes by a post-translational mechanism; an hypothesis is formulated here for the biogenesis of peroxisomes.**

Several laboratories have attempted to determine the intracellular site of synthesis of catalase (hydrogen peroxide:hydrogen peroxide oxidoreductase, EC 1.11.1.6) by various approaches (for review, see ref. 1). However, none of the reported results has provided clear-cut evidence as to whether catalase is synthesized on free ribosomes, on membrane-bound ribosomes, or on both.

In the present study we have investigated the intracellular site of synthesis of two peroxisomal enzymes of rat liver, catalase and uricase (urate:oxygen oxidoreductase, EC 1.7.3.3). Our data demonstrate that both peroxisomal enzymes are synthesized exclusively by free ribosomes. The implications of these results for the segregation of peroxisomal proteins within peroxisomes are discussed, and an hypothesis for the biogenesis of peroxisomes is proposed.

METHODS

Most of the procedures used in the present study have been detailed elsewhere. Among these are: the preparation, from rat liver, of free polysomes and of membrane-bound polysomes isolated from rough microsomes by detergent treatment in the presence of RNase inhibitor (2); the extraction of a total RNA fraction from rat liver or from free polysomes with sodium dodecyl sulfate ($NaDodSO_4$)/phenol/chloroform/isoamyl alcohol (3, 4); the preparation of a poly(A)-containing mRNA fraction by affinity chromatography on oligo(dT)-cellulose (3); the preparation, from isolated dog pancreas rough microsomes, of ribosome-denuded "microsomal membranes" by the EDTA procedure (5); the assay for cell-free protein synthesis by using a staphylococcal nuclease-treated rabbit reticulocyte lysate (6); and various post-translational assays, including determination of hot acid-insoluble radioactivity (3), incubation with proteolytic enzymes (6), polyacrylamide gel electrophoresis in $NaDodSO_4$ (6), and fluorography of unstained gels (7).

Preparation of Antisera. Commercial bovine catalase (Calbiochem, La Jolla, CA) and porcine uricase (Sigma Chemical Co., St. Louis, MO) were used as antigens. One milligram of protein was dissolved in 1 ml of 0.15 M NaCl/0.01 M dithiothreitol/0.5% $NaDodSO_4$. After incubation in a boiling water bath for 2 min and subsequent cooling to room temperature, 50 μmol of α-iodoacetamide was added and incubation was continued for 1 hr at 37°. Aliquots of these samples containing up to 50 μg of antigen were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis; both antigens were more than 95% pure as estimated from the Coomassie brilliant blue staining intensity of the banding pattern (data not shown). Aliquots containing 250 μg of antigen were emulsified with complete Freund's adjuvant, and rabbits were injected intraperitoneally three times at weekly intervals. Blood was withdrawn beginning 2 weeks after the last injection; booster injections in incomplete Freund's adjuvant were administered as necessary. Antibodies to bovine catalase and porcine uricase crossreacted with rat liver uricase and catalase preparations (see below) as assayed by Ouchterlony double-diffusion analysis.

Preparation of Rat Liver Uricase and Catalase by Cell Fractionation and Immunoprecipitation. Livers from rats fasted overnight were homogenized in 0.25 M sucrose/0.05 M Tris·HCl, pH 7.5/0.05 M KCl/5 mM $MgCl_2$ as described (2).

Uricase. We took advantage of the fact that peroxisomal cores (which contain uricase) are not solubilized by sodium deoxycholate. A crude cell fraction containing peroxisomes was prepared by centrifuging a postnuclear supernatant (10 min, 1000 × *g*) for 15 min at 25,000 × *g*. The resulting pellet was resuspended in 0.25 M sucrose/0.05 M Tris·HCl, pH 7.5/0.05 M KCl/5 mM $MgCl_2$ and the suspension was incubated at 0° for 5 min with sodium deoxycholate at a final concentration of 1%. Subsequent centrifugation for 10 min at 25,000 × *g* yielded a pellet which, by the criterion of $NaDodSO_4$/polyacrylamide gel electrophoresis, consisted primarily of one polypeptide (data not shown) of approximately 31,000 daltons, or slightly larger than porcine uricase which migrates as a polypeptide of approximately 30,000 daltons in our gel system. The material in this pellet was dissolved in 5% $NaDodSO_4$ and labeled with sodium [^{125}I]iodide by the chloramine-T procedure (8). Iodinated uricase was purified by immunoprecipitation using monospecific antiserum and staphylococci, exactly as described below for uricase synthesized *in vitro*.

Catalase. Unlike uricase, catalase is not part of the sodium deoxycholate-insoluble peroxisomal core, and a significant

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Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; ER, endoplasmic reticulum.

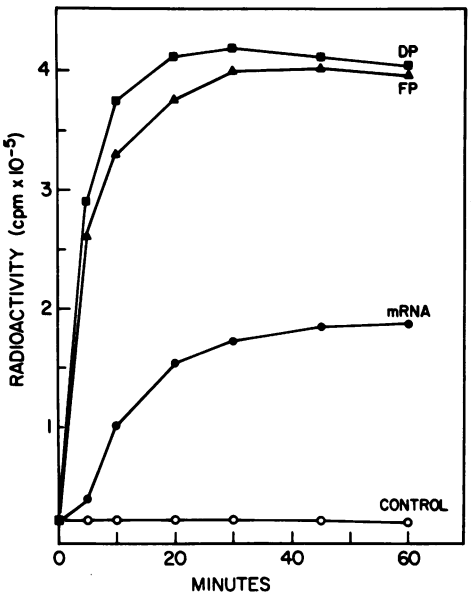

FIG. 1. Time course of protein synthesis in a nuclease-treated reticulocyte lysate cell-free system programmed with various rat liver fractions. Each 50-μl reaction mixture (6) contained 25 μCi of [^{35}S]-methionine and either 1.25 A_{260} units of membrane-bound polysomes (DP), 1.25 A_{260} units of free polysomes (FP), 0.05 A_{260} unit of poly(A)-containing mRNA (mRNA), or none of the above (control). Incubations were performed at 29°. Hot acid-insoluble radioactivity of 5-μl aliquots was determined (3) at the times indicated.

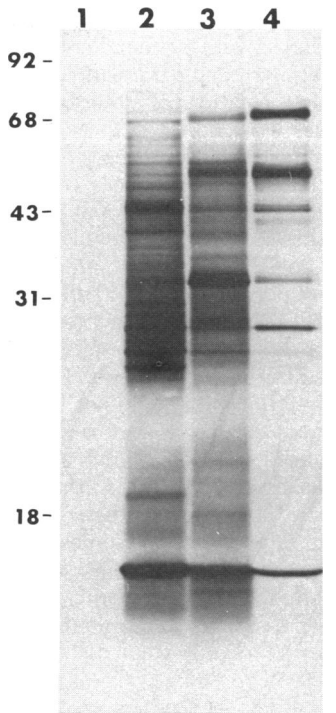

FIG. 2. Analysis by NaDodSO$_4$/polyacrylamide gel electrophoresis and subsequent fluorography of the products synthesized in the experiment described in Fig. 1. Aliquots (of indicated volumes) were analyzed in each case from the 60-min time point of incubation (Fig. 1). Lanes: 1, control (1 μl); 2, free ribosomes (1 μl); 3, membrane-bound ribosomes (1 μl); 4, mRNA (2 μl). The numbers to the left of lane 1 indicate the molecular weight, $\times 10^{-3}$, of marker proteins, and the bars next to the numbers indicate their electrophoretic mobilities. The following marker proteins were used: rabbit muscle phosphorylase B (92,000); bovine serum albumin (68,000); chicken ovalbumin (43,000); bovine pancreas DNase I (31,000); sperm whale myoglobin (18,000). Marker proteins were iodinated by the chloramine-T procedure (8).

amount of it can be found in a high-speed supernatant fraction, presumably due to peroxisome rupture during homogenization. To prepare radiolabeled catalase, we injected 1 mCi of [^{35}S]-methionine into the portal vein of an anesthetized 150-g male rat. After 10 min the liver was excised and homogenized (see above). A high-speed supernatant was prepared by centrifugation first for 10 min at 15,000 $\times$ g and then for 2 hr at 100,000 $\times$ g. Catalase was directly immunoprecipitated from the high-speed supernatant adjusted to contain 150 mM NaCl, 0.2% (wt/vol) Triton X-100, and 100 units of Trasylol per ml. After passage of the supernatant through a Millipore filter (0.22 μm), 1.5 vol of anticatalase antiserum was added. The mixture was incubated for 10 min at 37° and overnight at 4°. Immunoprecipitates were collected by centrifugation and washed three times with 10 mM Tris·HCl, pH 7.4/150 mM NaCl/5 mM EDTA/0.1% Triton X-100 containing 100 units of Trasylol and 1 μg of unlabeled methionine per ml. Final immunoprecipitates were solubilized in 4% NaDodSO$_4$. Analysis by NaDodSO$_4$/polyacrylamide gel electrophoresis and fluorography showed a single band at *ca* 60,000 daltons, which comigrated with catalase partially purified from rat liver by the "micropurification" method of Lazarow and de Duve (9).

Immunoprecipitation of Albumin, Catalase, and Uricase Synthesized *In Vitro*. Aliquots for immunoprecipitation (generally 50–200 μl; see figure legends) were taken after 60 min of incubation in the cell-free system (see legend to Fig. 1). NaDodSO$_4$ was added to a final concentration of 2%. The samples were incubated in a boiling water bath for 2 min and then cooled to room temperature before the addition of 4 vol of 190 mM NaCl/50 mM Tris·HCl, pH 7.4/6 mM EDTA/2.5% Triton X-100. After thorough mixing, 10 μl of antiserum was added and the mixture was incubated for 60 min at 37° and subsequently for 16 hr at 4°. Antigen–antibody complexes were precipitated with heat-inactivated and formaldehyde-fixed staphylococci according to Kessler (10), except for the presence in the wash buffer of 0.1% NaDodSO$_4$ instead of nonionic detergent.

Sources of Materials. [^{35}S]Methionine (specific activity, 700–1100 Ci/mmol) was purchased from Amersham/Searle (Arlington Heights, IL). Trypsin, chymotrypsin, staphylococcal nuclease, and proteinase K were obtained from Boehringer (Mannheim, Germany). Rabbit muscle phosphorylase B and chicken ovalbumin were purchased from Worthington Biochemicals (Freehold, NJ). Bovine serum albumin, bovine pancreas DNase I, and sperm whale myoglobin were from Sigma Chemical Co. (St. Louis, MO). Trasylol was obtained from Mobay Chemical Corp. (New York, NY). Heat-inactivated, formaldehyde-fixed *Staphylococcus aureus*, strain Cowan I (American Type Culture Collection 12598) was kindly provided by C. Chang. Rabbit antiserum to rat albumin was purchased from Cappel Laboratories (Cochranville, PA).

RESULTS

Free polysomes, membrane-bound polysomes prepared from detergent-treated rough microsomes, and total mRNA, all isolated from rat liver, were incubated in a staphylococcal nuclease-treated rabbit reticulocyte lysate system. The time course of incorporation of radioactive amino acids into hot acid-insoluble protein is shown in Fig. 1. It should be noted that the protein-synthesizing activities of free and bound polysomes were similar, thus allowing meaningful comparison of the products synthesized by these two populations of ribosomes.

Analysis of the synthesized products by NaDodSO$_4$/polyacrylamide gel electrophoresis (Fig. 2) showed striking dif-

ferences in the banding pattern between the products made by free polysomes (lane 2) and by bound polysomes (lane 3). The major bands resulting from the translation of total mRNA (lane 4) were present in the pattern of either free (lane 2) or bound (lane 3) polysomes.

As a more specific test of the efficacy of separation between free and membrane-bound polysomes, we determined by immunoprecipitation the relative amount of albumin that was synthesized by these two populations of polysomes. It can be seen from Fig. 3 and Table 1 that albumin was synthesized by membrane-bound polysomes (lane 2) and that it was not detectable among the products made by free polysomes (lane 1). These data are in agreement with results obtained recently in RNA·DNA hybridization experiments (11).

Immunoprecipitation with monospecific antisera prepared against catalase and uricase was next used to determine which of the two polysome populations synthesized these peroxisomal enzymes. The data in Fig. 3 and Table 1 show that catalase (lane 6) as well as uricase (lane 9) are synthesized by free polysomes and not by membrane-bound polysomes (lanes 7 and 10, respectively). Treatment with nonimmune serum as a control did not precipitate any major bands from among the translation products of either free (lane 3) or membrane-bound (lane 4) polysomes.

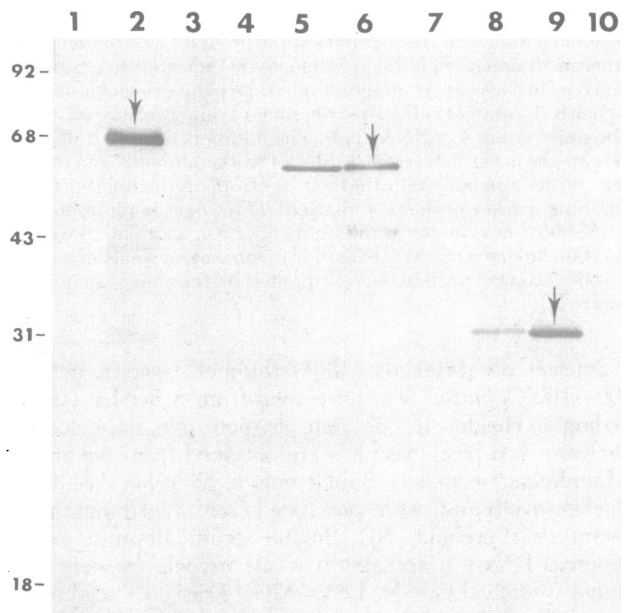

FIG. 3. Localization of the biosynthetic sites of albumin, catalase, and uricase. Aliquots from the cell-free synthesis mixture or from rat liver fractions were subjected to immunoprecipitation with monospecific antisera against rat albumin (lanes 1 and 2), bovine catalase (lanes 5–7), or porcine uricase (lanes 8–10) or with nonimmune serum (lanes 3 and 4). The volume of cell-free synthesis mixture immunoprecipitated in each case was 200 μl, except that in lanes 1 and 2 it was 50 μl. All of the immunoprecipitated material was analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and subsequent fluorography, except for lane 2, to which only one-third of the total immunoprecipitate was applied. Immunoprecipitates of products synthesized in the cell-free system by free polysomes are in lanes 1, 3, 6, and 9; immunoprecipitates of products of membrane-bound polysomes are in lanes 2, 4, 7, and 10. Lanes 5 and 8 contain immunoprecipitated, radiolabeled catalase and uricase, respectively, purified from rat liver fractions. Arrows indicate *in vitro* synthesized and immunoprecipitated albumin (lane 2), catalase (lane 6), and uricase (lane 9). Numbers and bars to the left of lane 1 are as in Fig. 2. The minor band in lane 6 migrating at approximately 45,000 daltons represents radioactivity nonspecifically associated with the immunoglobulin heavy chains.

Table 1. Quantitation of immunoprecipitated products synthesized in the cell-free system by free and membrane-bound polysomes

	Free polysomes		Membrane-bound polysomes	
Antiserum against	cpm	Lane	cpm	Lane
Albumin	60	1	1000	2
Catalase	340	6	40	7
Uricase	680	9	80	10

Bands indicated by arrows in lanes 2, 6, and 9 of Fig. 3 and of equivalent regions in adjacent lanes 1, 7, and 10 were excised from the dried and fluorographed gel and assayed for radioactivity directly in toluene/Liquifluor. Data have not been corrected for background, which ranged from 40 to 60 cpm, as determined by assay of gel slices excised from lanes that did not contain radioactivity.

It should be noted that the mobilities of uricase and catalase synthesized in the cell-free system by free polysomes were identical to those of uricase and catalase isolated from rat liver (Fig. 3). The same result was obtained when uricase (or catalase) was synthesized by translating phenol-extracted mRNA from free polysomes in the cell-free system (Fig. 4, lane 3). Thus, by the criterion of electrophoretic mobility in $NaDodSO_4$/polyacrylamide gels, larger precursors for uricase and catalase were not synthesized in our cell-free system (see *Discussion*).

Our finding that mRNA for both uricase and catalase is located in the free polysome fraction does not *per se* constitute proof that these enzymes are synthesized on free polysomes *in vivo*. It is conceivable, for example, that mRNAs for peroxisomal enzymes were found in the free polysomal fraction because they were temporarily disengaged from translation (e.g., in response to overnight fasting of rats or as a result of possible

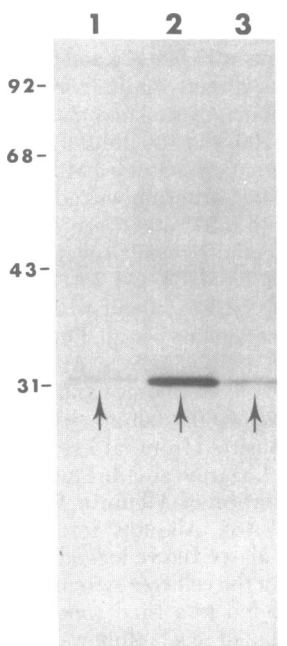

FIG. 4. Comparison of electrophoretic mobilities of purified rat liver uricase (lane 1) and of uricase synthesized in the cell-free system by rat liver free polysomes (lane 2) or by mRNA extracted from free polysomes (lane 3). Immunoprecipitates were derived from 200 μl (lane 2) or 400 μl (lane 3) of cell-free synthesis mixture. Rat liver uricase in lane 1 was prepared as in lane 8 of Fig. 3. Analysis was by polyacrylamide gel electrophoresis and subsequent fluorography. Arrows point to uricase. The slight upward shift of the uricase in lane 1 is an artifact due to iodination (data not shown). Numbers and bars to the left of lane 1 are as in Fig. 2.

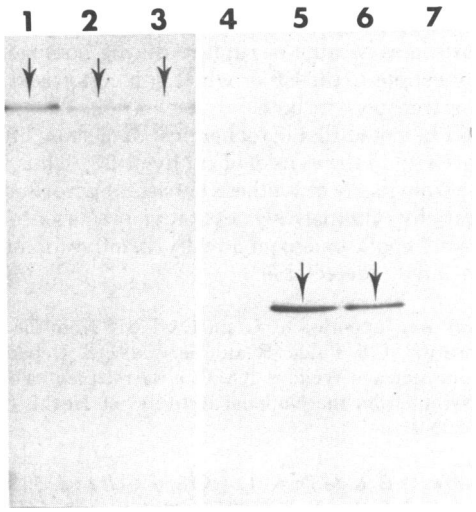

FIG. 5. Cotranslational segregation of albumin but not of uricase by dog pancreas microsomal membranes. Rat liver mRNA was translated in the cell-free system in the absence (lanes 1, 4, and 5) or in the presence (lanes 2, 3, 6, and 7) of nuclease-treated (6) microsomal membranes. Aliquots (lanes 3, 4, and 7) were assayed for segregation by post-translational incubation with trypsin and chymotrypsin (each at 300 μg/ml) for 2 hr at 4 °. Proteolysis was terminated by the addition of Trasylol (1000 units/ml). Samples were immunoprecipitated either with anti-albumin antiserum (lanes 1–3) or with anti-uricase antiserum (lanes 4–7). Lanes 1–3 and 4–7 were derived from two slab gels with composite alignment based on migration of marker proteins.

diurnal fluctuations in the synthesis of peroxisomal enzymes). One could therefore argue that when these mRNAs reengage in translation they would attach to endoplasmic reticulum (ER) membranes by the same mechanism that has been proposed for mRNAs of secretory proteins (3) and thus would be found in the membrane-bound ribosome fraction. To investigate this possibility, we translated total liver mRNA in the absence or presence of dog pancreas microsomal membranes and assayed for segregation by post-translational incubation with proteolytic enzymes (5). As expected, and as shown previously for a number of other secretory proteins (4, 5, 12), the newly synthesized albumin was segregated by dog pancreas microsomal vesicles (presumable as proalbumin), as evidenced by its resistance to proteolysis (Fig. 5, lane 3). By the same criterion, newly synthesized uricase was not segregated by the microsomal vesicles (Fig. 5, lane 7). Similar results were obtained for catalase (data not shown).

DISCUSSION

Peroxisomal enzymes represent one of several groups of proteins for which passage across an intracellular membrane is an obligatory step in the pathway from synthesis to enclosure within a membrane-bounded compartment. Two distinct mechanisms have so far been uncovered for the transfer of proteins across intracellular membranes (13). In "cotranslational" transfer, passage across the membrane is tightly coupled to translation and proceeds only *during* synthesis of the protein by membrane-bound ribosomes (3, 5). In "post-translational" transfer, passage through the membrane(s) occurs after protein synthesis and is *not* mediated by a ribosome–membrane junction (14). Both mechanisms, however, share conceptually common features. The information for membrane transfer is encoded in part of the newly synthesized protein as a "signal" sequence that in most cases is removed during or after passage across the membrane. There should be several structurally distinct signal sequences, different for co- and post-translational passage and specific for each of the intracellular membranes across which transfer proceeds. The signal sequences are presumably recognized by specific membrane receptors. Interaction of the signal sequences of the *nascent* chain (cotranslational transfer) or of the *completed* chain (post-translational transfer) with the membrane receptors has been proposed to result in the formation of a transient proteinaceous pore in the membrane through which the protein can pass (3, 13, 14).

Our present studies demonstrate that two peroxisomal enzymes, uricase and catalase, are synthesized by free ribosomes and not by membrane-bound ribosomes. Moreover, whereas newly synthesized albumin was found to be segregated in our cell-free system supplemented with microsomal membranes, uricase and catalase were not. These results were surprising because ultrastructural studies had shown that peroxisomes bud from the ER (15), suggesting a cotranslational transfer mechanism—i.e., synthesis of peroxisomal enzymes by ribosomes bound to the ER exactly as for secretory proteins, and a subsequent sorting mechanism operating within the ER lumen to separate peroxisomal enzymes from secretory proteins (13, 16). It is clear that such a pathway for peroxisomal enzymes can be ruled out by our present data.

Thus, unlike the case for secretory proteins, transfer of peroxisomal enzymes across the membrane must proceed by a post-translational mechanism. Our present data, however, do not resolve whether a short-lived signal sequence is involved in membrane passage, analogous to that used for post-translational transfer of protein from the cytosol into the chloroplast (14, 17, 18). Our attempts to achieve cell-free synthesis of a larger precursor for either uricase or catalase so far have failed. However, we argue that such precursors most likely do exist and that the failure to synthesize them *in vitro* is due to soluble signal peptidase in our cell-free system; this putative signal peptidase, specific for signal sequences of peroxisomal enzymes, would presumably be released from fragmented peroxisomes during preparation of the cell-free extract. Two lines of evidence from other systems support these conjectures. First, a precursor for a glyoxysomal enzyme, malate dehydrogenase, has recently been synthesized in a wheat germ cell-free system (19). Because glyoxysomes are the plant cell equivalents of animal cell peroxisomes (20–23), it is likely that similar precursors also are synthesized in animal cells. Second, a soluble signal peptidase (14) that is confined to the chloroplast in the intact cell (18), and that specifically cleaves the signal sequences that mediate post-translational cytosol–chloroplast transfer, is found in the high-speed supernatant of homogenized *Chlamydomonas reinhardtii* (14). This signal peptidase is absent from wheat germ cell-free extracts, permitting synthesis of precursor when *Chlamydomonas* mRNA is translated (14); however, when *Chlamydomonas* free ribosomes are incubated for cell-free protein synthesis, no precursor is found because of signal peptidase adventitiously bound to the ribosomes during their isolation (14). Because we did not observe synthesis of larger precursors of uricase or catalase even when the cell-free system was programmed with phenol-extracted mRNA, a signal peptidase for signal sequences of peroxisomal enzymes, if it exists, must also be present in the rabbit reticulocyte lysate. Similarly, it must also be present in our wheat germ extract, because translation of mRNA yielded mature-sized uricase only (data not shown). It is interesting that a small amount of peroxisomal (glyoxisomal)-type signal peptidase activity also may have been present in the wheat germ extract used to synthesize the larger precursor for malate dehydrogenase, because some mature form was also synthesized (19). Alternatively, it is possible that uricase and catalase contain uncleaved signal sequences and therefore are not synthesized as larger precursors.

Our evidence that uricase and catalase are synthesized exclusively by free ribosomes raises the question Why do peroxisomes arise as budding outgrowths of the ER? We propose that the connection between the ER and the peroxisomal membranes functions in the flow of certain integral proteins of the peroxisomal membrane from their site of synthesis and insertion at the rough ER. It has been shown recently that integral transmembrane proteins are inserted into the membrane cotranslationally using a ribosome membrane junction (24, 25). We therefore propose the following sequence of events for the biogenesis of peroxisomes and glyoxysomes.

After cotranslational insertion into the rough ER membrane, constitutive peroxisomal membrane proteins (including putative receptors specific for signal sequences of peroxisomal enzymes) are sorted from other integral transmembrane proteins by virtue of some structural information common only to peroxisomal membrane proteins ("sorting" sequence). Sorting should proceed by patching and capping in the plane of the membrane; when it is complete (the ultrastructural equivalent of the budding stage), import of newly synthesized peroxisomal enzymes from the cytosol begins, proceeding by a post-translational mechanism. A signal sequence that is characteristic for peroxisomal "content" proteins interacts with peroxisomal signal receptor proteins, forming a transient passageway through the newly assembled peroxisomal membrane for the unidirectional transfer, from the cytosol, of a single, newly synthesized, peroxisomal protein. Cleavage of the putative peroxisomal signal sequence by peroxisomal signal peptidase should occur during or shortly after passage and could be linked to the disassembly (or closing) of the passageway, thus completing the cycle for the transfer of a single protein molecule. Some mechanism must exist to suppress activity of the receptor while it is still in the rough ER, thus preventing premature transfer of peroxisomal content proteins into the cisternae of the rough ER. Similarly, a "lock" must prevent newly imported peroxisomal content proteins from diffusing back into the cisternae of the rough ER.

Our model for peroxisome biogenesis predicts that newly synthesized peroxisomal content proteins will be found in "nascent" peroxisomes that are in the process of budding from the ER. By virtue of their newly acquired receptors and their spatial capacities, these nascent peroxisomes would be the major, if not the only, site for import of content proteins. Loading of "nascent" peroxisomes may be completed within a short time. Mature peroxisomes would have lost the capacity for import, either because of receptor inactivation or because of spatial limitations.

Thus, synthesis of peroxisomal content proteins and of peroxisomal integral membrane proteins (at least of those that span the membrane) would proceed diffusely and at different intracellular sites, on free and membrane-bound ribosomes, respectively. The assembly of these proteins into peroxisomes, however, would proceed in a highly coordinated and synchronized manner at only a few foci, ultastructurally apparent as budding outgrowths of the ER and referred to here as "nascent" peroxisomes.

Compatible with our model are the reports of *in vivo* labeling experiments that found newly synthesized catalase in a high-speed supernatant fraction (1, 26). It is not clear, however, whether this newly synthesized catalase had leaked from nascent peroxisomes because of rupture during homogenization of the connections to the ER or whether it comprised catalase waiting for transport, or both.

It should be noted that cytochemical data show little or no catalase activity in the cytosol of rat liver (27). Thus, transfer of catalase from its site of synthesis to nascent peroxisomes may proceed rapidly. Alternatively, a putative precursor to catalase in the cytosol could be enzymatically inactive or otherwise cytochemically undetectable.

This work was supported by Grant CA-12413 from the National Cancer Institute, U.S. Public Health Service, U.S. Department of Health, Education and Welfare. B.M.G. is the recipient of a postdoctoral fellowship from the National Institutes of Health (Grant 1-F32-EY05208-01).

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