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Three Peroxisome Protein Packaging Pathways Suggested by Selective Permeabilization of Yeast Mutants Defective in Peroxisome Biogenesis

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We have identified five complementation groups of peroxisome biogenesis (*peb*) mutants in *Saccharomyces cerevisiae* by a positive selection procedure. Three of these contained morphologically recognizable peroxisomes, and two appeared to lack the organelle altogether. The packaging of peroxisomal proteins in these mutants has been analyzed with a new gentle cell fractionation procedure. It employs digitonin titration for the selective permeabilization of yeast plasma and intracellular membranes. Proteins were measured by enzymatic assay or by quantitative chemiluminescent immunoblotting. With this gentle fractionation method, it was demonstrated that two mutants are selectively defective in assembling proteins into peroxisomes. *Peb1-1* packages catalase and acyl-CoA oxidase within peroxisomes but not thiolase. *Peb5-1* packages thiolase and acyl-CoA oxidase within peroxisomes but not catalase. The data suggest that the peroxisome biogenesis machinery contains components that are specific for each of three classes of peroxisomal proteins, represented by catalase, thiolase, and acyl-CoA oxidase. In the two mutants lacking morphologically recognizable peroxisomes, *peb2-1* and *peb4-1*, all three enzymes were mislocalized to the cytosol.

INTRODUCTION

We have recently reported the isolation of new yeast mutants in which peroxisome biogenesis is defective (*peb*[1] mutants). In two complementation groups, *peb2* and *peb4*, peroxisomes were not found at all by electron microscopy nor by electron microscopic (EM) cytochemistry (Zhang *et al.*, 1993). These yeast mutants may be models of the human genetic disease, Zellweger syndrome, in which defects in peroxisome bio result in the absence of morphologically recognizable peroxisomes (Lazarow and Moser, 1989). In one complementation group, *peb3*, only a few peroxisomes, which displayed weak thiolase immunofluorescence, were detected.

Remarkably, in two mutant complementation groups normal-looking peroxisomes were present, but they occurred in clusters. These peroxisomes contained many, but not all, of the normal peroxisome proteins. The two groups differed in which of the peroxisomal proteins was missing according to EM cytochemistry and immuno-gold labeling. In mutant *peb5-1*, catalase was not detected in peroxisomes, but thiolase was present. In another complementation group, *peb1*, containing several mutants, the reverse was true. *Saccharomyces cerevisiae* mutants with similar defects in packaging thiolase or catalase have also been reported by van der Leij *et al.* (1992).

We have now investigated in more detail, using cell fractionation, the intracellular locations of three peroxisomal proteins in one mutant from each of our five *peb* complementation groups. These three proteins were selected because it was thought likely that they might have different topogenic signals. Calatase A, the peroxisomal catalase isozyme in *S. cerevisiae*, has an SKL-like C-terminus plus internal topogenic information (Kragler *et al.*, 1993). SKL and related tripeptides are responsible for targeting many proteins to peroxisomes (Gould *et al.*, 1990b) in a variety of species (Gould *et al.*, 1990a). Thiolase is

[1] Abbreviations used: AOx, acyl-CoA oxidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *pas*, peroxisome assembly; *peb*, peroxisome biogenesis; PGK, phosphoglycerate kinase.

directed to peroxisomes by a cleavable N-terminal peptide, at least in rat liver (Osumi *et al.*, 1991; Swinkels *et al.*, 1991). Acyl-CoA oxidase (AOx) in *S. cerevisiae* contains neither an SKL-like C-terminus nor a thiolase-like N-terminus. We speculated that it might be targeted to peroxisomes by internal peptides as it is in *Candida tropicalis* (Small *et al.*, 1988). As controls, the intracellular distributions of several enzymes belonging to other cell compartments were also analyzed.

To obtain reliable data, we had to overcome a drawback of current fractionation procedures for studying yeast peroxisomes. The problem was that peroxisomes are fragile and tend to be damaged during homogenization, centrifugation, and resuspension of fractions (McCammon *et al.*, 1990; Thieringer *et al.*, 1991) causing leakage of the contents. Therefore, a more gentle procedure was sought.

In the case of animal cells, plasma membranes and intracellular membranes can be selectively permeabilized by digitonin on the basis of their content of cholesterol (de Duve, 1965; Wanders *et al.*, 1984). Digitonin forms a stoichiometric complex with cholesterol producing holes in the membrane. The concentration of digitonin needed to permeabilize a membrane is inversely proportional to the abundance of sterol in that membrane. The plasma membrane, which is richest in cholesterol (Colbeau *et al.*, 1971), is permeabilized by low concentrations of digitonin; this releases cytosolic proteins from the cells. The lysosome membrane contains less cholesterol and requires a higher concentration of digitonin to allow the lysosomal enzymes to leak out. The mitochondrion and peroxisome, which contain little if any cholesterol in their membranes, require the highest concentrations of digitonin (de Duve, 1965; Wanders *et al.*, 1984); in this case the digitonin may be functioning as a nonspecific detergent. Thus, the intracellular locations of enzymes can be determined by analyzing the extent of their release from cells as a function of the digitonin concentration applied. An important advantage of this method is that it avoids mechanical breakage of the organelles and the attendant artefactual release of organelle proteins.

S. cerevisiae does not contain cholesterol in its membranes but does contain a related sterol, ergosterol (Longley *et al.*, 1968; Rattray *et al.*, 1975). We speculated that digitonin might also form complexes with ergosterol and thus permeabilize yeast membranes. Here we report the successful use of digitonin for the selective permeabilization of yeast cell membranes. We have analyzed the specificity of the protein packaging defects in our *peb* mutants and found evidence for a three-pronged pathway of protein import into peroxisomes.

MATERIALS AND METHODS

Yeast Strains and Media

The wild-type yeast strain JW68 (*α ura3-1 trp1-1 arg4 ctt1-1*) and five *peb* mutants (*peb1-1 a ura3-1 trp1-1 ctt1-1; peb2-1 α ura3-1 trp1-1 arg4 ctt1-1; peb3-1 α ura3-1 trp1-1 arg4 ctt1-1; peb4-1 α leu2-3 leu2-112 ura3-1 trp1-1 arg4 ctt1-1; peb5-1 a leu1 trp1-1 arg4 ctt1-1*) have been described (Zhang *et al.*, 1993). The mutants, which were generated with ethyl methane sulfonate, were backcrossed to wild-type cells one or more times. In every case, the Peb phenotype (as assessed by immunofluorescence and growth on oleic acid) was recessive and segregated 2:2. The original strain subjected to mutagenesis lacked catalase activity (cta, ctt). A wild-type CTA gene for the peroxisomal isozyme of catalase, catalase A, was reintroduced into each mutant strain by the backcrossing.

The yeast were precultured twice in rich, glucose-containing medium (YPD) (Sherman *et al.*, 1986) and then grown in medium containing glycerol and oleic acid to induce peroxisomes (Zhang *et al.*, 1993). This induction medium contained 1% (wt/vol) yeast extract, 2% (wt/vol) bacto-peptone, 3% (wt/vol) glycerol, 0.1% (wt/vol) oleic acid and 0.25% (vol/vol) Tween 40.

Digitonin Titration

Cells were harvested by centrifugation at 1500 rpm for 5 min. The cell mass was weighed and washed twice with buffer A (100 mM tris(hydroxymethyl)aminomethane-Cl pH 7.4, 50 mM EDTA). The cells were resuspended in 50 ml of buffer A containing 10 mM β-mercaptoethanol and incubated at 30°C for 20 min with shaking. The cells were pelleted by centrifugation, resuspended in 6 volumes (of cell mass) of SP buffer (1.2 M sorbitol, 20 mM potassium phosphate buffer pH 7.4) plus Zymolyase 100-T (ICN Biomedicals, Costa Mesa, CA), and incubated at 30°C for 45 min with gentle shaking (60–100 rpm). For wild-type cells, 2 mg of Zymolyase/g cell mass was used. Some mutants required less Zymolyase and shorter times for good cell wall digestion. As little as 0.5 mg/g cell mass and 10 min of incubation was used in some cases. The formation of spheroplasts was monitored carefully under the microscope. When 80–90% of the cells had been converted to spheroplasts, the digestion was stopped by the addition of an equal volume of ice-cold SP buffer. They were harvested by centrifugation at 1700 rpm for 5 min at 4°C and washed twice with 10 volumes of ice-cold SP buffer. The spheroplasts were then resuspended in 5 volumes of ice-cold SP buffer containing a mixture of protease inhibitors (0.7 mM each of chymostatin, antipain, pepstain, and leupeptin, and 20 mg/ml phenylmethylsulfonyl fluoride [PMSF]). Ethanol (0.1%) was added to protect catalase activity (Chance, 1950).

Aliquots of the spheroplast suspension (900 μl) were added to a series of tubes containing digitonin dissolved in 100 μl of SP buffer. The amounts of digitonin were chosen to obtain the final concentrations shown in Figure 2B. The tubes were mixed gently by inversion and incubated at 4°C for 20 min with rotation. After the incubation, the mixture was centrifuged in an Eppendorf microcentrifuge (Westbury, NY) at 14 000 rpm for 15 min at 4°C. The supernatants were recovered and were ready for further analysis.

Total cell homogenates were prepared by adding ~400 mg of glass beads to a tube containing 900 μl of yeast spheroplast suspension, vortexing for 5 min at 4°C, and centrifuging for 5 min at 5000 rpm. The supernatants were collected for analysis.

Western Blots

Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes according to standard methods (Harlow and Lane, 1988). The Enhanced Chemiluminescence Western blotting system from Amersham (Arlington Heights, IL) was used for the immunodetection of proteins according to the company's protocol. X-OMAT film from Kodak (Rochester, NY) was used to record the signal. The films were scanned and converted to digitalized images with a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA). Scanning was conducted with 12-bit A/D converters to permit accurate quantitation (Nelson and Wygant, 1992) of the two-dimensional images. The in-

tegrated protein band intensities were computed with the ImageQuant software (Molecular Dynamics) of the Personal Densitometer. In preliminary experiments we found conditions where the signal was proportional to the antigen concentration over a reasonable range for each of the three antibodies (anti-thiolase, anti-AOx, and anti-phosphoglycerate kinase [PGK]) (Figure 1). This method had another advantage in the case of the antiserum against AOx that reacted with two minor bands in addition to the oxidase (Figure 1A); it was easy to quantitate just the major band. These linear immunoblotting assay conditions were used in all of the experiments reported here. The immunoblot images were printed on an XL7700 continuous tone printer (Kodak).

Assays

Fumarase (Bergmeyer *et al.*, 1983b) and glyceraldehyde-3-phosphate dehydrogenase (GADPH) (Bergmeyer *et al.*, 1983a) were assayed as described. Catalase was measured according to Baudhuin *et al.* (1964). Protein concentrations were determined by the Bio-Rad (Richmond, CA) dye-binding assay with bovine immunoglobulin as a standard (Bradford, 1976).

Materials

Rabbit antiserum against *S. cerevisiae* acyl-CoA oxidase was a gift from Dr. Joel Goodman (University of Texas). Rabbit anti-yeast thiolase was kindly provided by Dr. Wolf Kunau (University of Bochum, Germany). Rabbit anti-yeast phosphoglycerate kinase was a gift from Dr. Jeremy Thorner (University of California, Berkeley). Digitonin (D1407, ~50% by thin-layer chromatography analysis according to the company; in this study it was assumed to be 100%) was from Sigma (St. Louis, MO). Glycerol-3-phosphate was from Boehringer Mannheim (Mannheim, Germany). Molecular mass standards were from GIBCO, BRL (Grand Island, NY).

RESULTS

Cell Fractionation of S. cerevisiae by Selective Permeabilization of Its Membranes with Digitonin

We tested, on wild-type *S. cerevisiae*, the feasibility of selectively permeabilizing plasma and intracellular membranes with digitonin. Wild-type yeast were grown in glycerol plus oleic acid to induce peroxisomes and were converted to spheroplasts as described in MATERIALS AND METHODS. The spheroplasts were treated with different concentrations of digitonin as indicated in Figure 2. Two cytosolic enzymes, GAPDH that was measured enzymatically and PGK that was quantitated by immunoblotting, were released from cells

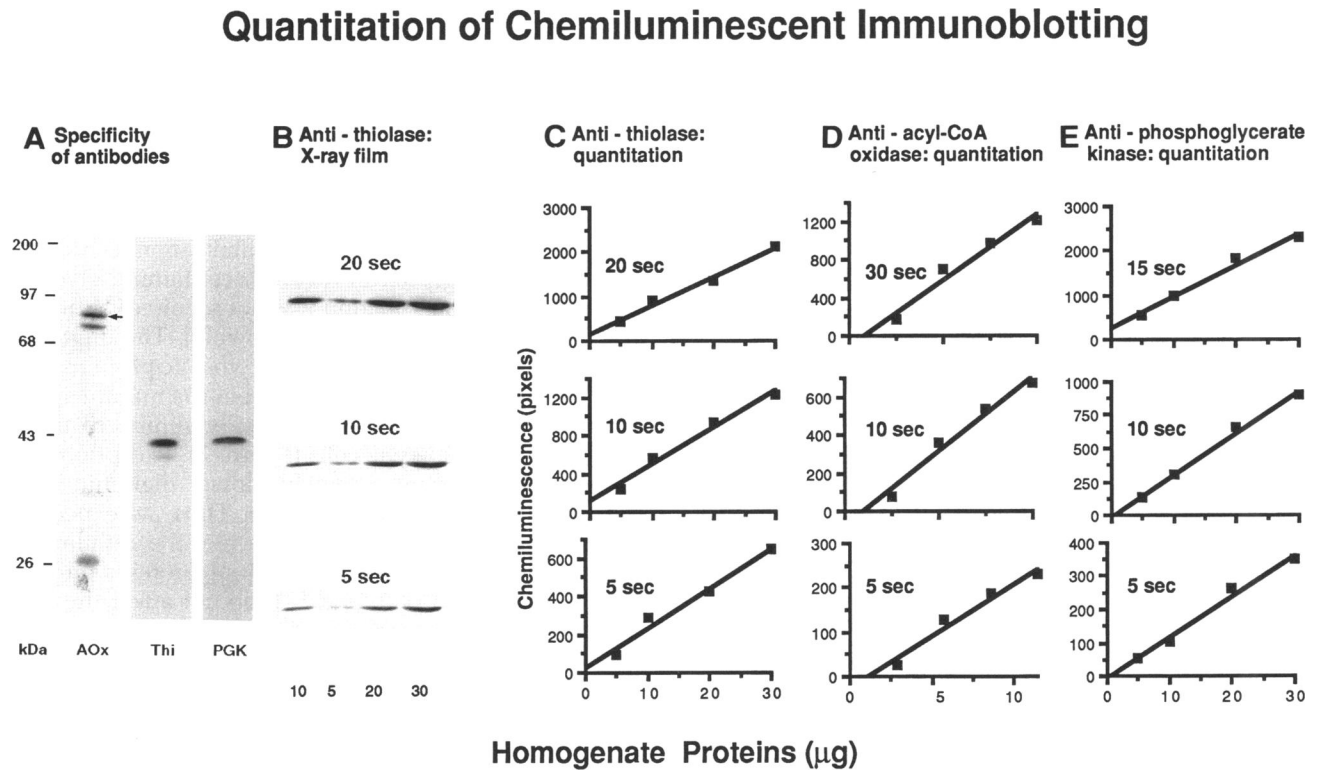

Figure 1. Quantitative detection of proteins by chemiluminescent immunoblotting. (A) Specificity of antibodies. Yeast extracts (20 μg of protein of total cell homogenates) were separated by SDS-PAGE, blotted with antisera against AOx, thiolase (Thi), and PGK. The antibodies were detected by chemiluminescence, which was recorded on X-ray film, as described in MATERIALS AND METHODS. Arrow indicates AOx. (B) Effect of the amount of homogenate protein (5–30 μg) and the exposure time on the chemiluminescent signal from anti-thiolase. Digitized image of scanned film printed on gray-scale printer. (C–E) Quantitation of the chemiluminescence intensity by laser densitometry of the X-ray film and computerized integration of band densities. (C) Quantitation of thiolase data of B. (D) AOx quantitation. (E) Phosphoglycerate kinase quantitation. Chemiluminescence exposure times are indicated in the figure.

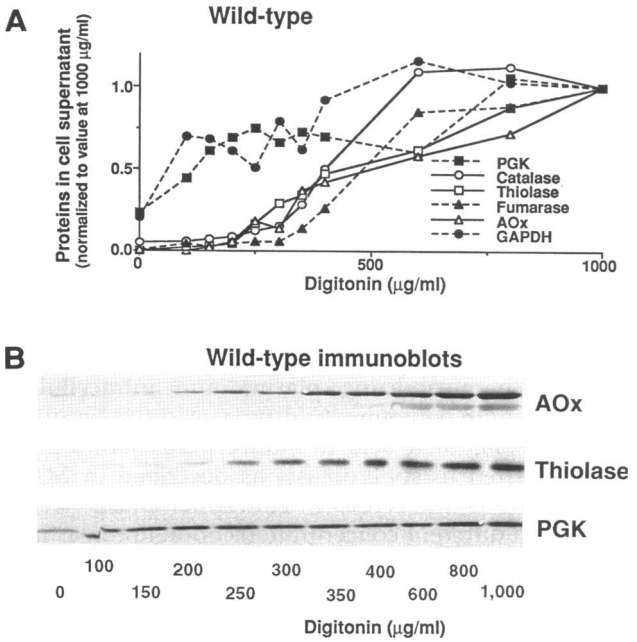

Figure 2. Release of proteins from wild-type yeast spheroplasts by digitonin titration. Aliquots of spheroplasts were incubated with digitonin at the concentrations indicated for 20 min at 4°C and then were centrifuged to pellet the cells. Peroxisomal enzymes: catalase, thiolase, and AOx. Mitochondrial enzyme: fumarase. Cytosolic enzymes: GAPDH and PGK. The activities of GAPDH, catalase, and fumarase in the supernatants were assayed as described in MATERIALS AND METHODS. The amounts of PGK, thiolase, and AOx protein in the supernatants were determined by immunoblotting as shown in Figure 1. (A) Plot of the amount of each enzyme released, normalized to the amount found in the supernatant at 1000 μg/ml of digitonin. (B) Raw immunoblot data. Each antiserum was applied to a separate nitrocellulose blot. The relevant section of each film is shown as a computerized image.

in parallel (Figure 2, ●, ■). Substantial amounts of both enzymes appeared in the supernatant at the lowest concentration of digitonin tested, 100 μg/ml. Release was ~70% complete at 200 μg/ml. With the exception of one data point (at 600 μg/ml), the data for the two cytosolic enzymes agreed closely, as they should for two enzymes that are being released from the same intracellular compartment.

Fumarase, a marker enzyme for the mitochondrial matrix, remained in the spheroplasts at the low concentrations of digitonin that released cytosolic enzymes. Fumarase was released at ~600 μg/ml. This difference in the concentrations of digitonin that were required to permeabilize the plasma membrane and the mitochondrial membranes was reproducible and sufficient to clearly distinguish experimentally between enzymes in the cytosol and enzymes in the mitochondria.

Catalase A was used as a marker enzyme for peroxisomes. It is the only active catalase isozyme in the strains used here (Cohen *et al.*, 1985; Zhang *et al.*, 1993). Catalase A was released to the supernatant at concentrations of digitonin similar to those required for mitochondrial enzymes (Figure 2). Two other peroxisomal enzymes, thiolase and AOx, emerged together with catalase, as would be expected if their release depended on permeabilization of the same membrane. Each of the three peroxisomal enzymes began to emerge slightly before fumarase (Figure 2), and this small difference appeared consistently in other experiments. Control experiments with total cell homogenates prepared with glass beads demonstrated that release of all enzymes was essentially complete at 1 mg/ml of digitonin (Zhang, unpublished data). These data indicate that digitonin may be applied to the selective permeabilization of *S. cerevisiae* membranes.

Peroxisomal Protein Expression in the Peb Mutants

The abundance of peroxisomal proteins was quantified in the mutant cells (Figure 3). There was no significant change in the amount of catalase, thiolase, or AOx in *peb* mutants *1-1*, *2-1*, *4-1*, or *5-1*. Significantly less catalase activity and thiolase protein were found in *peb3*. AOx protein was present in *peb3* at levels close to those in wild-type cells.

Application of the Digitonin Permeabilization Method to Peroxisome Biogenesis Mutants of S. cerevisiae

The subcellular locations of the three peroxisomal proteins in our five peroxisome biogenesis mutants were analyzed with the digitonin permeabilization technique. The yeast were grown in medium containing glycerol (so that peroxisome function was not required) and oleic acid (to induce peroxisomes if possible). The digitonin concentrations needed to release cytosolic proteins and to release mitochondrial protein from the mutant spheroplasts (Figures 4–6) were generally similar to those required with wild-type cells (Figure 2). Exceptionally, fumarase emerged at somewhat lower digitonin concentrations in *peb1-1* (Figure 5A). Thus, as expected, these five *peb* mutations do not affect the cellular topology of cytosolic or mitochondrial proteins and, at least in *peb2-1*, *3-1*, *4-1*, and *5-1*, do not affect the susceptibility of the mitochondrial and plasma membranes to digitonin. The change in fumarase's behavior in *peb1-1* needs to be further investigated in a null mutant to determine whether it is a consistent property of *peb1* mutations.

Distribution of Peroxisomal Enzymes in Peb Mutants in Which Peroxisomes Are Not Recognizable Morphologically

Peroxisomes were not found in mutants *peb2-1* and *peb4-1* despite extensive morphological searching

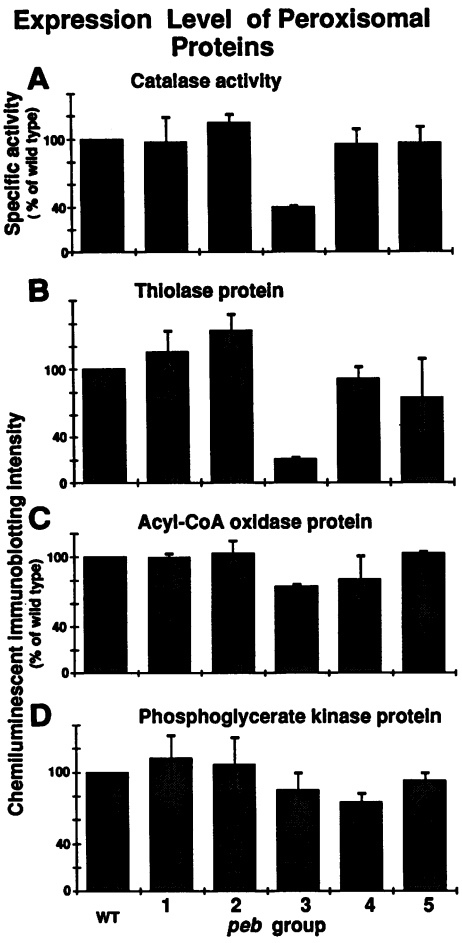

Figure 3. Expression levels of peroxisomal proteins in wild-type and mutant yeast cells. Three separate experiments were carried out, and the results are expressed as the means. Error bars indicate the standard deviations. Total cell homogenates were prepared with glass beads as described in MATERIALS AND METHODS. Catalase was measured enzymatically. This activity belongs to the peroxisomal isozyme, catalase A. The gene for catalase T, a cytosolic isozyme in *S. cerevisiae*, is nonfunctional in these cells (Cohen *et al.*, 1985). Thiolase, AOx, and PGK were determined by quantitative immunoblotting and densitometry. Equal amounts of homogenate protein from wild-type and each mutant were subjected to SDS-PAGE. The amount of each protein found in the mutants is represented as the percentage of that in wild-type cells. In the case of AOx, the error bars represent the range in two experiments.

(Zhang *et al.*, 1993). Digitonin treatment of these two mutants caused the release of catalase, thiolase, and AOx together with the cytosolic marker enzyme PGK (Figure 4). These data demonstrate that the three peroxisomal enzymes are mislocated to the cytosolic compartment in these cells. This is consistent with the morphological absence of peroxisomes and with the known facts of peroxisome biogenesis. Peroxisomal proteins are synthesized on free polyribosomes from which they are released into the cytosol when synthesis is complete (Lazarow and Fujiki, 1985); without peroxisomes, they remain in the cytosol.

Distribution of Peroxisomal Enzymes in Peb Mutants in Which Peroxisomes Are Present

***peb1-1* Mutant.** Thiolase and catalase were released separately from *peb1-1* spheroplasts by digitonin (Figure 5A, compare O and □). Thiolase emerged with the cytosolic marker PGK. Catalase came out at digitonin concentrations slightly lower than those that release fumarase. These data demonstrate that catalase is packaged within peroxisomes, but thiolase is not.

AOx was released at approximately the same digitonin concentrations as catalase, consistent with a peroxisomal localization. A portion of both of these enzymes was found within the supernatants at the low digitonin concentrations where cytosolic proteins emerge, raising the possibility that packaging may be incomplete, leaving ~20–30% of these peroxisomal proteins in the cytosol.

The data shown represent the means of two entirely separate experiments. The modest sizes of the error bars, which indicate the range of values in individual experiments, demonstrate the reproducibility of the digitonin permeabilization technique and the consistent behavior of the mutant.

***peb5-1* Mutant.** Catalase cofractionated with the cytosolic proteins in this mutant (Figure 5B, O). AOx be-

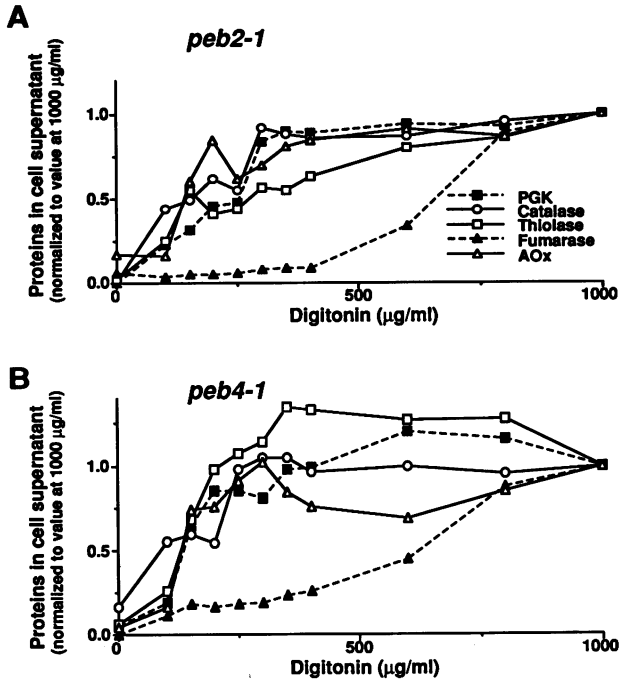

Figure 4. Digitonin titration: mutants with no recognizable peroxisomes. (A) *peb2-1*. (B) *peb4-1*.

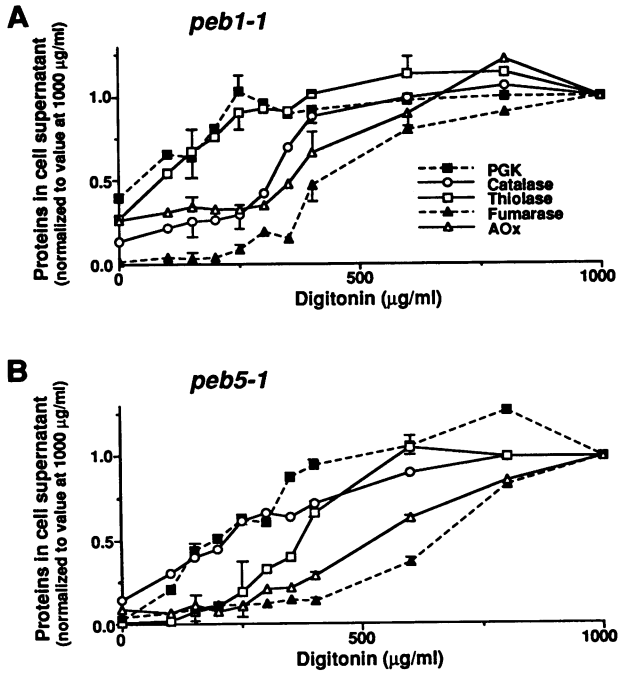

Figure 5. Digitonin titration: mutants that contain peroxisomes. (A) *peb1-1*. (B) *peb5-1*. Each plot represents the mean values of two entirely separate experiments. The range of the individual values is indicated for digitonin concentrations of 150, 250, 400, and 600 µg/ml. For the sake of clarity, only the upper or lower half of an error bar is shown. Where error bars are not visible at these digitonin concentrations, they lie within the symbol.

haved exactly as in wild-type cells, emerging just before the mitochondrial marker (△). Unexpectedly, thiolase was largely released between 250 and 400 µg/ml of digitonin. This is a greater digitonin concentration than is required for cytosolic proteins but lower than for AOx. The error bars demonstrate the reproducibility of these results in two experiments. Thiolase was unambiguously found in peroxisomes in this mutant by immunogold labeling (Zhang *et al.*, 1993). The significance of the release of thiolase at an intermediate digitonin concentration will be discussed below.

Selective Packaging Defects

These fractionation data demonstrate that mutants *peb1-1* and *peb5-1* contain some but not all of the peroxisomal proteins within the peroxisomes. Thiolase is left in the cytosol in *peb1-1*, whereas catalase is left in the cytosol in *peb5-1*. Both of these proteins contain the correct targeting information to be packaged into peroxisomes. Catalase is expressed from a wild-type gene that was put into the mutants by backcrossing, and this catalase is correctly imported into peroxisomes in two of the mutants. Thiolase of *peb1* is imported into peroxisomes in a diploid cell formed by mating *peb1* with a yeast strain lacking thiolase (Zhang *et al.*, 1993). Therefore, these mutations must affect the packaging mechanism not the targeting information.

AOx appears to be assembled normally into peroxisomes in both selective packaging mutants. These data suggest that the import of AOx involves a mechanism that differs from the one used by catalase and differs from the one used by thiolase.

Distribution of Peroxisomal Enzymes in a Peb Mutant with Lower Expression of Some Peroxisomal Proteins

The steady-state expression levels of catalase and thiolase were lower in *peb3-1* than in wild-type cells, whereas the expression of AOx was approximately normal (Figure 3). All three peroxisomal proteins were released together at digitonin concentrations between 200–400 µg/ml (Figure 6). This is more digitonin than is needed for cytosolic proteins but less than for the mitochondrial proteins. It is also less digitonin than is needed for release of peroxisomal proteins in wild-type cells. The intermediate amount of digitonin required to solubilize all three peroxisomal proteins in *peb3-1* was similar to the amount required to release thiolase in *peb5-1*. These and other data suggest that the properties of the peroxisome membrane may have been altered (see DISCUSSION).

DISCUSSION

Implications for Peroxisome Biogenesis

Mutant *peb3-1* has two distinct abnormalities in its peroxisomal phenotype. Two peroxisomal proteins (at least) were not induced normally by growth on oleate. One of these proteins is thiolase, which explains the faint thiolase immunofluorescence in the peroxisomes that was observed previously (Zhang *et al.*, 1993). All three peroxisomal enzymes that were tested demonstrated the same dependence on digitonin concentration for their release from the spheroplasts, which is

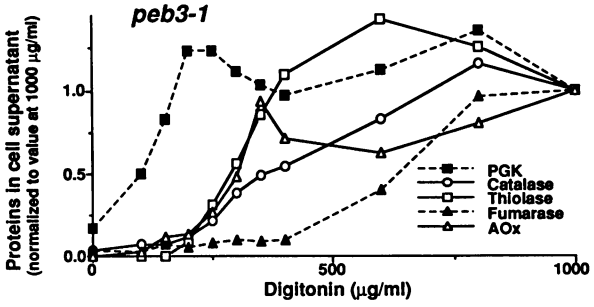

Figure 6. Release of proteins from mutant *peb3-1*, which has a low expression level of some peroxisomal proteins, by digitonin titration.

consistent with their location within peroxisomes. The peroxisomal localization was confirmed for thiolase in *peb3-1* by immunoelectron microscopy (Zhang *et al.*, 1993). Unexpectedly, the amount of digitonin that was required to solubilize the three peroxisomal enzymes was distinctly less than the amount required to solubilize the mitochondrial marker enzyme, in contrast to wild-type cells (Figure 6). This suggests that the properties of the peroxisomal membrane, perhaps its ergosterol content or perhaps some other characteristic, may be altered in *peb3-1*. One may speculate that this alteration in membrane properties could be the result of the lack of induction of some other peroxisomal enzyme(s). This will be explored further in the future on a null mutant.

All peroxisomal proteins tested in mutants *peb2*-1 and *peb4-1* were found in the yeast cytosol (Figure 4). This confirms and extends our previous immunofluorescence, cytochemical, and immunolocalization experiments in which peroxisomes were not seen (Zhang *et al.*, 1993). It is consistent with the fact that peroxisomal proteins are imported posttranslationally into preexisting peroxisomes from the cytosol (Lazarow and Fujiki, 1985). No mislocalization of peroxisomal proteins to other organelles was detected.

Digitonin titration of mutants *peb1-1* and *peb5-1* demonstrated the mislocalization to the cytosol of individual peroxisomal proteins (Figure 5). In the case of *peb1-1*, thiolase fractionated with the cytosolic marker enzymes, whereas the other two peroxisomal enzymes emerged just before the mitochondrial marker, as in wild-type cells. These biochemical results agree with our morphological data.

Each of the three peroxisomal enzymes was released from *peb5-1* spheroplasts in a distinct fashion (Figure 5B). Catalase emerged with the cytosolic proteins, consistent with the previous observation that there was no catalase cytochemical reaction product in peroxisomes (Zhang *et al.*, 1993). AOx responded to digitonin as it did in wild-type cells, emerging just before the mitochondrial marker enzyme. Thiolase release, unexpectedly, was intermediate between the two other peroxisomal enzymes and distinctly different from both. Could thiolase be mislocalized to some other organelle? No, it was found within peroxisomes by immunogold labeling (Zhang *et al.*, 1993). Could AOx be mislocalized to some other organelle? This appears unlikely in view of the fact that 1) the release of the oxidase from *peb5-1* appears to be indistinguishable from its wild-type behavior and 2) when AOx is not packaged into peroxisomes in other mutants, it is found in the cytosol.

If thiolase and AOx are both located in peroxisomes in *peb5-1*, why do they emerge at different concentrations of digitonin? One possibility is that there is some difference in the forces retaining them within the organelle as digitonin begins to permeabilize the peroxisomal membrane. In the case of rat liver, the oxidase remains partially associated with peroxisomes that have been damaged by freezing and thawing or by low concentrations of Triton X-100, whereas catalase and thiolase are easily released from damaged peroxisomes. This is thought to be the result of the binding of the oxidase to other proteins within the peroxisomal matrix. The oxidase is released from rat liver peroxisomes by higher concentrations of Triton X-100 (Alexson *et al.*, 1985). A similar mechanism might explain the data of *peb5-1*. A protein with which thiolase normally associates might be missing from peroxisomes in the *peb5* mutant so that thiolase leaks out more easily. Whatever the explanation, it must account for the fact that no differential leakage of peroxisome enzymes was observed from wild-type spheroplasts or from *peb3-1* spheroplasts. All three peroxisomal enzymes emerged together from these strains. Further experiments are required in the future to investigate this question.

It is noteworthy that the data on the mutants with selective packaging defects suggest that there are at least three distinct pathways, or three branches in a pathway, for the import of proteins into peroxisomes (Figure 7). Among catalase, thiolase and AOx, the import of either of the first two could be abolished without affecting the import of the others. One possibility is that these three proteins may have different topogenic features and use different receptors. *S. cerevisiae* thiolase may use an amino-terminal targeting sequence like that of rat liver thiolase (Osumi *et al.*, 1991; Swinkels *et al.*, 1991). Catalase has a carboxyl-terminal SSNSKF (a variant of SKL) that is sufficient for import in *S. cerevisiae* but is not required for import (Kragler *et al.*, 1993). The topogenesis of *S. cerevisiae* AOx is unknown. It lacks an SKL-like carboxyl-terminus, and its amino-terminal sequence does not ap-

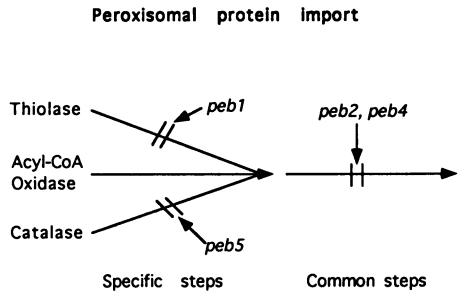

Figure 7. Speculative model of peroxisome biogenesis suggested by the observed packaging defects. *Peb2* and *peb4* prevent the packaging of all three enzymes whereas *peb1* and *peb5* each affect just one. One possibility is that the *PEB1* and *PEB5* genes encode receptors and the *PEB2* and *PEB4* genes encode parts of the translocation machinery. The temporal and topological relationships of these gene products are unknown.

pear to resemble that of thiolase (Dmochowska *et al.*, 1990). Perhaps it contains internal targeting peptides like AOx from *C. tropicalis* (Small *et al.*, 1988). Thus, one interpretation of our data is that there may be three distinct receptors that recognize peroxisomal targeting features, one for each of the three peroxisomal proteins tested. Whether the observed selective packaging defects are because of mutations in these putative receptors or to other defects remains to be elucidated by further experiments.

New Techniques

The application of increasing concentrations of digitonin proved to be an effective method to selectively permeabilize the membranes of *S. cerevisiae*. Two cytosolic enzymes were released together at low digitonin concentrations. Three peroxisomal enzymes were released together from wild-type peroxisomes at substantially higher digitonin concentrations. Fumarase was released from mitochondria at an even higher digitonin concentration (Figure 2). We assume that digitonin probably functions by forming complexes with ergosterol in the yeast membrane, although that has not been demonstrated in these experiments. Regardless of mechanism, the digitonin titration of yeast spheroplasts permits one to determine the intracellular location of enzymes without the mechanical disruption associated with conventional cell fractionation methods.

Moreover, because digitonin gently permeabilizes the plasma membrane without affecting the integrity of organelles, it could be used for in vitro studies of protein import into organelles in yeast. Digitonin permeabilization of mammalian cells has been used successfully for in vitro import assays of nuclear proteins (Adam *et al.*, 1990) and for studies of endosome acidification and function (Diaz and Stahl, 1989).

Another technological advance in this study was that conditions were found for the linear assay of proteins on Western blots, exploiting the sensitivity of the chemiluminescence detection procedure (Figure 1). The application of chemiluminescence detection to immunoblotting has greatly increase the sensitivity of antigen measurements. However, to the best of our knowledge, this is the first instance in which the quantitative aspects of this procedure have been systematically tested. The observed linearity permits quantitative determination of antigens.

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