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Peroxisomes in *Saccharomyces cerevisiae*: Immunofluorescence Analysis and Import of Catalase A into Isolated Peroxisomes

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To isolate peroxisomes from *Saccharomyces cerevisiae* of a quality sufficient for in vitro import studies, we optimized the conditions for cell growth and for cell fractionation. Stability of the isolated peroxisomes was monitored by catalase latency and sedimentability of marker enzymes. It was improved by (i) using cells that were shifted to oleic acid medium after growth to stationary phase in glucose precultures, (ii) shifting the pH from 7.2 to 6.0 during cell fractionation, and (iii) carrying out equilibrium density centrifugation with Nycodenz containing 0.25 M sucrose throughout the gradient. A concentrated peroxisomal fraction was used for in vitro import of catalase A. After 2 h of incubation, 62% of the catalase was associated with, and 16% was imported into, the organelle in a protease-resistant fashion. We introduced immunofluorescence microscopy for *S. cerevisiae* peroxisomes, using antibodies against thiolase, which allowed us to identify even the extremely small organelles in glucose-grown cells. Peroxisomes from media containing oleic acid were larger in size, were greater in number, and had a more intense fluorescence signal. The peroxisomes were located, sometimes in clusters, in the cell periphery, often immediately adjacent to the plasma membrane. Systematic immunofluorescence observations of glucose-grown *S. cerevisiae* demonstrated that all such cells contained at least one and usually several very small peroxisomes despite the glucose repression. This finding fits a central prediction of our model of peroxisome biogenesis: peroxisomes form by division of preexisting peroxisomes; therefore, every cell must have at least one peroxisome if additional organelles are to be induced in that cell.

The biogenesis of microbodies (peroxisomes, glyoxysomes, and glycosomes) is characterized by certain features that distinguish them from other cell organelles. In eucaryotes, peroxisomes are nearly ubiquitous organelles that are surrounded by a single bilayer membrane. Matrix and membrane proteins of peroxisomes are synthesized on free polyribosomes and posttranslationally integrated into preexisting organelles by a mechanism that requires ATP hydrolysis. The precursor polypeptides of these proteins are in general synthesized at their mature size, and there is no proteolytic processing connected to uptake. A membrane protein (or proteins), which likely functions as a receptor, is required for import. New peroxisomes are generated by division after growth of the organelle (5, 24, 25).

Investigators have made many efforts to use eucaryotic microorganisms as model systems. In these systems, the study of the biogenesis of various cell organelles and of intracellular protein traffic has been facilitated by the ease of genetic regulation in response to environmental changes and by genetic manipulation with modern techniques of molecular biology (15). The yeast *Saccharomyces cerevisiae* has been genetically characterized in enormous detail and has already been used as a powerful tool of cell biology (32, 41, 42). Unlike the situation for other yeasts and filamentous fungi, peroxisomes of *S. cerevisiae* have not yet been characterized in great detail. Avers and Federman first demonstrated the presence of peroxisomes in yeast cells (2). Under the conditions chosen for growth and isolation, however, peroxisomes appeared to be rare, small, and very fragile. The presence of two different catalases in *S. cerevisiae* has been shown: catalase T (typical) and catalase A (atypical). The vacuolar (not peroxisomal) nature of catalase A has been discussed (43), although recent reports strongly suggest the peroxisomal localization of this protein (19, 35). Catalase T presumably is a cytosolic protein (43). The genes of both proteins have been cloned (7, 40) and sequenced (8, 20). Attempts to induce the proliferation of peroxisomes in *S. cerevisiae* failed for a long time, but recently Veenhuis and co-workers demonstrated that growth on oleic acid led to a pronounced induction of peroxisomal enzymes and proliferation of peroxisomes (46).

We characterized the appearance of peroxisomes under several growth conditions by immunofluorescence, electron microscopy, and immunoelectron microscopy. It is a widely held impression that *S. cerevisiae* cells generally lack peroxisomes, because random sectioning of glucose-grown cells usually does not show peroxisomal structures. By immunofluorescence microscopy, we demonstrate that even in glucose-repressed cells, there are at least one and generally several peroxisomes, confirming the predictions drawn from our current model of peroxisome biogenesis.

The importance of the choice of the growth conditions for the successful use of peroxisomes in an in vitro import assay has been previously shown in our laboratory for another yeast, *Candida tropicalis*. In this yeast, peroxisomes have been induced with Brij 35, resulting in the induction of a moderate number of medium-size peroxisomes, which appeared to be more stable after isolation than peroxisomes isolated after induction with oleic acid (38). However, our existing protocols for the isolation of *C. tropicalis* peroxisomes did not work for *S. cerevisiae*. Therefore, to study the targeting of peroxisomal proteins in *S. cerevisiae*, we inves-

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tigated the conditions for growth of the yeast and fractionation of its cell organelles to obtain a peroxisomal fraction useful for an in vitro import assay. We expressed the coding region of the *CTA1* gene of *S. cerevisiae* in vitro, and the resulting polypeptide, catalase A, was used for import in our in vitro system. Association and import occurred in a time- and temperature-dependent fashion.

MATERIALS AND METHODS

Materials. The following substances were obtained from the sources mentioned in parentheses: yeast extract and Bacto-Peptone (Difco Laboratories, Detroit, Mich.); Nycodenz (Accurate Chemical & Scientific Corp., Westbury, N.Y.); Zymolyase 100T and Tran^{35}S-label (ICN Immuno-Biochemicals, Lisle, Ill.); fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G, alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G, and restriction enzymes (Boehringer GmbH, Mannheim, Federal Republic of Germany); SP6 polymerase and pGEM-4 vector (Promega Biotec, Madison, Wis.). Other substances were obtained from Sigma, St. Louis, Mo.

A rabbit antiserum against 3-oxoacyl coenzyme A (3-oxoacyl-CoA) thiolase from *S. cerevisiae* was a generous gift of W.-H. Kunau, Ruhr-Universität, Bochum, Federal Republic of Germany. The antiserum is monospecific for thiolase by immunoblotting (23a) and is specific for peroxisomes in immunoelectron microscopy (18). Rabbit antisera against catalase A and catalase T from *S. cerevisiae* (49) were kindly provided by H. Ruis, University of Vienna, Vienna, Austria. Polyclonal antisera raised against total peroxisomal proteins of *C. tropicalis* (38) and rat liver 3-oxoacyl-CoA thiolase (12) have been described previously.

Growth and culture conditions. *S. cerevisiae*, wild-type D273-10B (ATCC 24657) and the catalase T-deficient mutant strain DCT1-2C (α *leu1 arg4 cas1 ctt1-1*; J. Rytka, Polish Academy of Science, Warsaw, Poland), were precultured at 30°C in 50 ml of medium containing 1% (wt/vol) yeast extract, 2% (wt/vol) Bacto-Peptone, and 2% (wt/vol) D-glucose (YPD) to late exponential growth phase. The cells were then transferred to 500 ml of medium (10^6 cells per ml) consisting of 1% (wt/vol) yeast extract, 2% (wt/vol) Bacto-Peptone, and one of the following carbon sources: 2% (wt/vol) D-glucose (YPD), 2% (wt/vol) ethanol (YPE), or 3% (wt/vol) glycerol (YPG).

For induction on oleic acid, we used two methods. For induction with growth, cells were precultured on YPD as described above to late exponential growth phase (18 h) and transferred to 500 ml of medium (10^6 cells per ml) containing 1% (wt/vol) yeast extract, 2% (wt/vol) Bacto-Peptone, 0.5% (wt/vol) oleic acid, and 0.3% (wt/vol) Brij 35 (YPOB). For induction with little growth, cells were grown to late stationary growth phase in 200 ml of YPD (40 h). The total cell mass of this preculture was transferred to 500 ml of a medium containing 0.3% (wt/vol) yeast extract, 0.5% (wt/vol) Bacto-Peptone, 30 mM K_2HPO_4, 0.15% (wt/vol) oleic acid, and 0.015% (vol/vol) Tween 40, adjusted to pH 6.0 (YPOT; approximately 2×10^7 cells per ml).

In all media, cells were incubated at 30°C on a rotary shaker.

Preparation of cell extracts. Cells were harvested by centrifugation, washed twice with distilled water, and then shock frozen with liquid nitrogen. A 400-mg aliquot of frozen cells was vigorously vortexed for 6 min with 3.5 g of glass beads (0.5 mm in diameter) in 1 ml of a buffer containing 50 mM Tris hydrochloride (pH 7.4), 2 mM EDTA, 2 mM ethylene glycol-bis-(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM dithiothreitol, 0.12 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% (vol/vol) ethanol, and the following protease inhibitors (prepared in dimethyl sulfoxide) at a final concentration of 0.7 mM each: chymostatin, antipain, pepstatin, and leupeptin. Vortexing was in 30-s intervals with cooling on ice in between. Glass beads and cell debris were removed by centrifugation (4 min, 2,500 rpm, 600 × *g*, Sorvall SS-34 rotor) through a column of glass wool in a syringe. The filtrate was taken as the total cell extract.

Preparation of spheroplasts. Cells were first incubated in 100 mM Tris hydrochloride (pH 7.4)–50 mM EDTA–10 mM β-mercaptoethanol for 20 min and then treated with Zymolyase 100T in 20 mM potassium phosphate buffer (pH 7.4) containing 1.2 M sorbitol for approximately 45 to 60 min at 30°C. We used 0.1 mg of Zymolyase per g (wet weight) of cells for the glucose-grown cells. 0.5 mg/g for ethanol- and glycerol-grown cells, and 2 mg/g for YPOT-induced cells.

Homogenization of spheroplasts and differential centrifugation. Spheroplasts were resuspended in ice-cold MSKE medium (5 mM 2-[*N*-morpholino]ethanesulfonic acid [MES; pH 6.0], 0.6 M sorbitol, 1 mM KCl, 0.5 mM EDTA, 0.1% [vol/vol] ethanol, 0.7 mM each chymostatin, antipain, pepstatin, and leupeptin). We used 1 ml of MSKE per g (weight prior to conversion to spheroplasts) of cells. Homogenization was carried out at 4°C in a Potter-Elvehjem homogenizer. Unbroken cells, cell debris, and nuclei were removed by centrifugation at 3,500 rpm (1,500 × *g*) for 5 min in a Sorvall SS-34 rotor. The pellet was resuspended in MSKE, rehomogenized, and centrifuged. The postnuclear supernatants were combined and centrifuged for 15 min at 15,000 rpm (25,000 × *g*). The resulting pellet (crude organelle fraction) was gently resuspended in 6 ml of MSKE, and a final centrifugation was carried out at 2,500 rpm (600 × *g*) for 5 min to remove larger aggregates.

For preparation of a crude organelle fraction at pH 7.2, a buffer containing 2.5 mM 3-[*N*-morpholino]propanesulfonic acid (MOPS; pH 7.2), 5% (wt/vol) Ficoll 400, 0.6 M sorbitol, 1 mM EDTA, 0.1% ethanol, and 0.7 mM each protease inhibitor mentioned above (MFSE) was used instead of MSKE.

Density gradient centrifugation. The following linear density gradients were used for the separation of cell organelles: (i) 30 to 50% (wt/wt) sucrose with a 1-ml cushion of 60% (wt/wt) sucrose in 2.5 mM MOPS (pH 7.2)–0.5 mM EDTA–0.1% (vol/vol) ethanol (gradient solution I) or 5 mM MES (pH 6.0)–1 mM EDTA–0.1% (vol/vol) ethanol (gradient solution II); (ii) 17 to 36% (wt/vol) Nycodenz resting on a 1-ml cushion of 42% (wt/vol) Nycodenz in 5 mM Tris hydrochloride (pH 7.5)–3 mM KCl–0.3 mM EDTA–0.1% ethanol (gradient solution III); and (iii) 15 to 36% (wt/vol) Nycodenz containing a uniform concentration of 0.25 M sucrose in gradient solution II or III. The gradients were centrifuged at 4°C in a Sorvall vertical rotor (Vt865B) at 35,000 rpm (100,000 × *g*; slow acceleration mode) for 60 min after reaching maximal speed.

Peroxisomal peak fractions were combined and diluted fivefold with a buffer containing 0.5 M sucrose, 1 mM EDTA, 5 mM MES, and 0.1% (vol/vol) ethanol, pH 6.0 (SEM). After centrifugation at 15,000 rpm (25,000 × *g*) at 4°C for 15 min, the pellet was resuspended in SEM to give a final protein concentration of 25 mg/ml.

Mitochondria, used as control organelles in in vitro import experiments, were obtained from cells grown on YPD. The crude organelle fraction was centrifuged into a 15 to 36% Nycodenz gradient containing 0.25 M sucrose (pH 6). The

mitochondrial peak fractions were concentrated as described for peroxisomes.

Immunofluorescence. Spheroplasts were resuspended in MFSE (20 ml/g of cells), and 200 μl of this suspension was transferred to a slide, previously covered with alcian blue, forming squares of approximately 1.5 by 1.5 cm. Slides were dried for 1 to 2 h, and the attached cells were permeabilized with methanol for 10 min. Squares were overlaid with 100 mM potassium phosphate buffer (pH 7.4)–0.9% (wt/vol) NaCl (PBS) containing 1% (wt/vol) bovine serum albumin and incubated for 30 min. The solution was carefully decanted onto filter paper and covered with 50 μl of rabbit antiserum diluted 1:150 with 1% (wt/vol) bovine serum albumin in PBS. After 2 h of incubation, the antiserum was decanted, and the spheroplasts were washed three times for 5 min with PBS. The squares were covered with FITC-conjugated to goat anti-rabbit immunoglobulin G (diluted 1:500 in PBS) and incubated for 1 h. At this step, all incubations were carried out protected from light. Samples were again washed three times for 5 min with PBS and mounted in 90% (vol/vol) glycerol in 100 mM Tris hydrochloride (pH 9.0). A Nikon Labophot epifluorescence microscope and a Bio-Rad confocal microscope were used for analysis and documentation.

Electron microscopy. Electron microscopy of spheroplasts and isolated organelles was carried out as described previously (38). For immunolabeling with protein A-gold, spheroplasts were fixed with 0.2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and embedded in Lowicryl-K_4M medium (31). For immunolabeling of peroxisomes, the organelles were fixed with 3% paraformaldehyde and 3% glutaraldehyde in SEM. Peroxisomes were detected with antiserum and 10-nm protein A-gold particles on ultrathin sections, which were stained with uranyl acetate.

Transcription and translation of catalase A and import into peroxisomes. The coding region of the catalase A gene (*CTA1*) (8) was cloned into pGEM-4 as described elsewhere (19a). The gene was transcribed with SP6 polymerase (28) after linearization of the plasmid with *Hin*dIII. The RNA was extracted with phenol-chloroform, ethanol precipitated, and translated in vitro with wheat germ extract (11) and Tran^{35}S-label. Sucrose (2.0 M) was added to the translation mixture to give a final concentration of 0.5 M sucrose. An in vitro import assay mixture (final volume, 35 μl) contained 15 μl of the adjusted translation mixture, 10 μl of concentrated peroxisomes (250 μg of protein in SEM), 50 mM KCl, and the following protease inhibitors (prepared in dimethyl sulfoxide) at a final concentration of 0.7 mM each: chymostatin, antipain, pepstatin, and leupeptin (38). Assay mixtures were incubated at 26°C for 1, 30, or 120 min. After incubation, three aliquots of 10 μl were taken, of which two were used for the protease protection assay. These samples were treated with 5 μg of proteinase K (which is not inhibited by the four inhibitors mentioned above) at 0°C for 30 min in the presence or absence of detergent (1% [wt/vol] deoxycholate and 1% [vol/vol] Triton X-100). The protease reaction was stopped by the addition of 1 mM PMSF. The mixtures were diluted eightfold with SEM containing 50 mM KCl, and the supernatants and peroxisome pellets were then separated by centrifugation at 16,000 × *g* in a microcentrifuge. The pellets were solubilized in SEM containing 1% (wt/vol) deoxycholate, 1% (vol/vol) Triton X-100, 0.7 mM each protease inhibitor (see above), and 1 mM PMSF. Equal amounts of each fraction were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), fluorography, and densitometric scanning. All reactions were carried out in siliconized 500-μl microtubes.

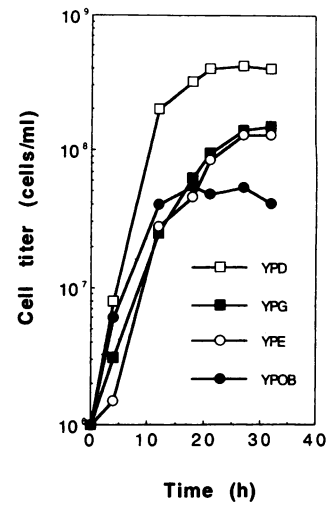

FIG. 1. Growth of *S. cerevisiae* on various carbon sources. Cells were grown at 30°C in 500 ml of medium containing glucose (YPD), glycerol (YPG), ethanol (YPE), or oleic acid (YPOB). Samples were taken at the times indicated to determine the cell titer with a hemacytometer.

Other methods. The following enzymes were assayed as described in the indicated references: catalase (3), cytochrome oxidase (26), NADPH-cytochrome *c* oxidoreductase (4), and isocitrate lyase (9). Alkaline phosphatase was measured in 0.1 M glycine buffer (pH 8.4)–10 mM $MgCl_2$–0.1% Triton X-100, using 6 mM *p*-nitrophenylphosphate as the substrate. The nitrophenol produced was measured at 405 nm, and a molar absorbance of 18,300 was assumed (48). Lauroyl-CoA was used as the substrate for the acyl-CoA oxidase assay (36).

Protein was determined by the Bio-Rad dye-binding assay with bovine immunoglobulin as the standard (6). SDS-PAGE and immunoblotting were carried out as previously described (37) except that we used alkaline phosphatase conjugated to goat anti-rabbit immunoglobulin G for immunodetection. The densities of the gradient fractions at 20°C were calculated from their refractive indices.

RESULTS

Growth of yeast cells and induction of peroxisomal enzymes. *S. cerevisiae* D273-10B could be grown with oleic acid as a carbon source (YPOB) with a cell yield of 4.5 g (wet weight)/liter. Growth stopped by 18 h (Fig. 1). Growth on oleate ended somewhat sooner than on glycerol (YPG) or ethanol (YPE), where stationary phase was observed after 26 h, and a cell yield of 6 g/liter was obtained. The cell yield for glucose-grown cells (YPD) was 14 g/liter of medium.

Acyl-CoA oxidase activity in cells grown on glucose was extremely low, whereas a shift to the oleate-containing YPOB medium led to a profound increase in this activity (Table 1). The specific activity of the oxidase rose steadily throughout the exponential growth phase (Fig. 2). Acyl-CoA oxidase activities in crude extracts of ethanol- or glycerol-grown cells were about 15- to 20-fold higher than in the glucose-grown cells but still represented only 3 to 5% of the specific activity obtained after growth on oleic acid (Table 1A).

TABLE 1. Induction of peroxisomal enzymes, catalase, and acyl-CoA oxidase[a]

Carbon Source	Concn (mU/mg)	
	Catalase	Acyl-CoA oxidase
A. Growth		
Glucose	15	5
Ethanol	6	75
Glycerol	16	97
Oleic acid	61	1,885
B. Induction with minimal growth		
Oleic acid	77	2,317

[a] Cells were grown to late exponential phase (A) or induced in YPOT for 18 h (B). The cells were broken with glass beads, and the enzymes were assayed in the clarified cell extracts.

We also studied the induction of peroxisomal enzyme activities when cells were shifted into oleic acid medium at high cell density such that cell growth was minimal during the following 18 h (YPOT, Table 1B). Induction of acyl-CoA oxidase was approximately the same as for cells grown on oleate.

The effect of the carbon source on catalase activity was less pronounced than for acyl-CoA oxidase. The highest levels of catalase activity were found in oleic acid-grown or -induced cells (Table 1). Catalase activity in cells grown on glucose was almost undetectable during early or midlogarithmic growth and rose modestly toward the end of exponential growth (not shown).

The interpretation of the apparent lesser induction of catalase than of acyl-CoA oxidase is complicated by the presence in *S. cerevisiae* of two catalases, A (34) and T (33), which are independently regulated (21). In a control experiment, we used a catalase T-deficient mutant of *S. cerevisiae* (DCT1-2C) (35). Catalase activity in glucose-grown cells was extremely low (less than 10% the level of the wild-type strain D273-10B). Induction with oleic acid led to a 200-fold increase in catalase activity (75% that of D273-10B cells). Induction of catalase in cells in oleic acid-containing media showed different kinetics than did other peroxisomal marker enzymes (Fig. 2). Whether this is due to the presence of the two different catalases in *S. cerevisiae* or to different mechanisms of induction needs further investigation.

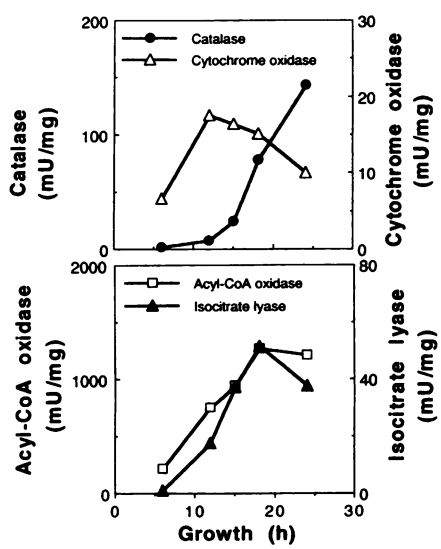

FIG. 2. Induction of enzyme activities during growth in YPOB. Cells were grown for the times indicated, harvested, and broken with glass beads. Protein and enzyme activities were determined on the clarified cell extracts.

The catalase activity of yeast cells grown in YP medium supplemented with 0.5% oleate but without Brij 35 detergent was approximately the same as for YPOB-grown cells. On the other hand, growth of cells in a medium containing 0.6% Brij 35 (which possesses a lauryl residue [C_{12} fatty acid]) but no oleate did not lead to induction of catalase activity in *S. cerevisiae* (data not shown). This contrasts with results for *C. tropicalis*, in which peroxisomes may be induced either by fatty acid alone or by Brij 35 alone (38).

Immunofluorescence. A few tiny peroxisomes (one to six per cell) could be visualized by immunofluorescence in *S. cerevisiae* grown on glucose to late exponential phase (Fig. 3, YPD). After growth on glycerol (Fig. 3, YPG) or ethanol (Fig. 3, YPE), we counted approximately the same number of peroxisomes per cell, but the immunofluorescence signal was considerably stronger, and the peroxisomes appeared to be larger. The peroxisomes of cells grown on oleic acid (Fig. 3, YPOB) or induced in stationary phase with oleic acid (Fig. 3, YPOT) were greater in number (6 to 13 per cell), looked larger, and definitely had a fluorescence signal more intense than those of the other growth conditions. A better resolution of the fluorescent signals was achieved by using a Bio-Rad confocal microscope. The micrograph shown in Fig. 4 was taken from the same slide which was prepared for epifluorescence analysis (shown in Fig. 3, YPOB). With the help of optical sectioning and image analysis, the fluorescence background of the samples could be significantly reduced, and the peroxisomes appeared as intensely fluorescent entities. The peroxisomes were often observed in the periphery of oleate-induced cells (Fig. 4).

Electron microscopy. To study the morphological consequences of growth of *S. cerevisiae* on different carbon sources in more detail, we carried out electron microscopy (Fig. 5). Peroxisomes were identified unambiguously by immunoelectron microscopy with protein A-gold particles (Fig. 5, inserts). A polyspecific antiserum raised against *C. tropicalis* total peroxisomal proteins cross-reacted with *S. cerevisiae* peroxisomes (Fig. 5, inserts). An antiserum against rat liver thiolase also cross-reacted (not shown).

Peroxisomes in cells grown on glucose were extremely rare in thin sections. In a survey of a large number of cells, we were able to identify presumptive peroxisomes, but we did not observe any immunolabeling in these glucose-grown cells and therefore could not confirm these identifications. In glycerol-grown cells, peroxisomes were also rare and had diameters less than 0.2 μm; their identity was confirmed by immunolabeling (Fig. 5B).

Peroxisomes in yeast cells grown on oleic acid were larger (approximately 0.2 to 0.4 μm in diameter) and more abundant. Often they were observed in the proximity of the plasma membrane (Fig. 5C). However, we did not see as massive a proliferation of peroxisomes as we and others have seen in *C. tropicalis* grown on oleate (38).

Isolation of yeast peroxisomes. To isolate peroxisomes that were pure and stable enough for use in in vitro import assays, we tried many isolation procedures. The morphological and biochemical results described above suggested that we could expect only moderate yields of peroxisomes from oleate-induced cells. Our initial attempts to isolate peroxisomes were frustrating: the conditions which in our labora-

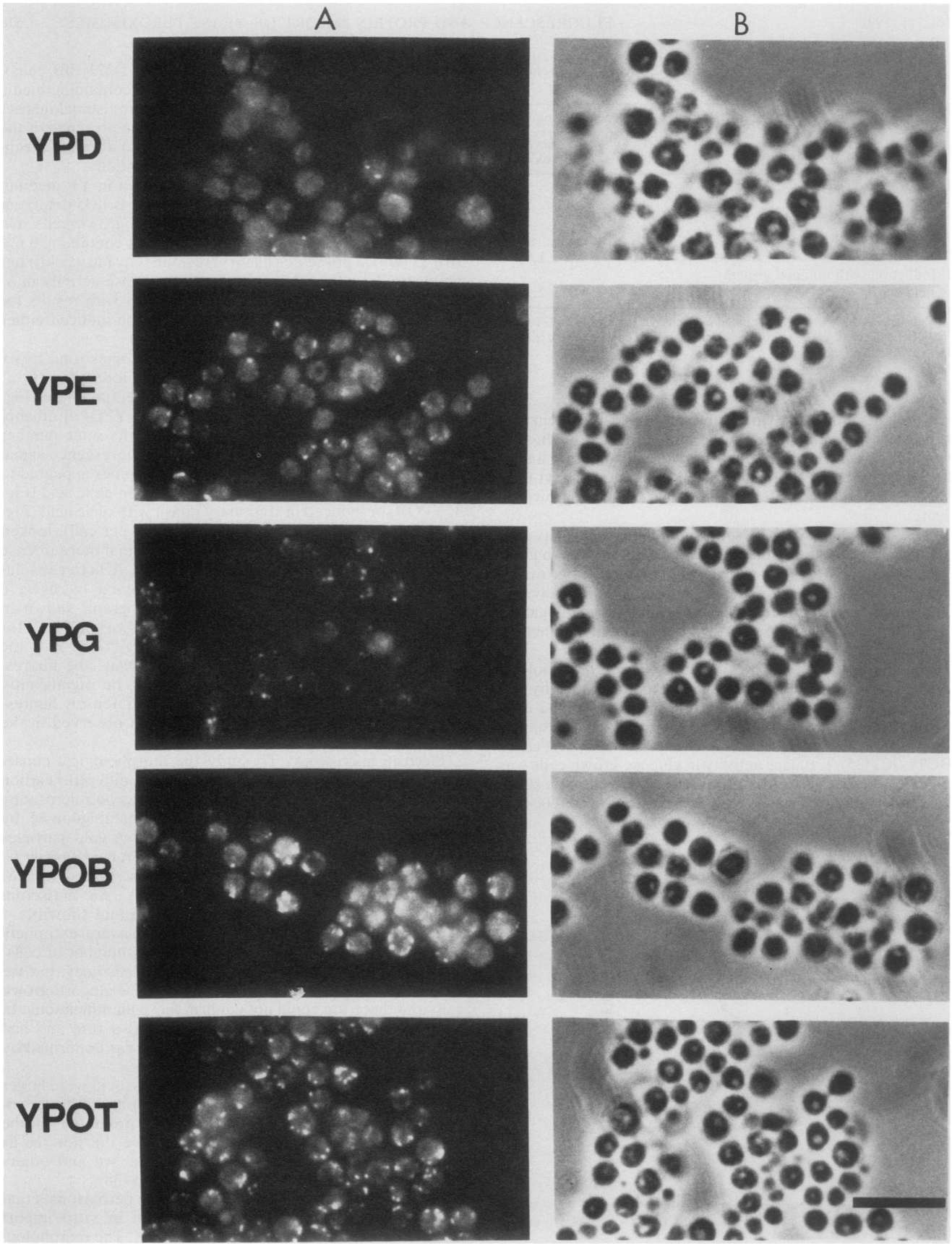

FIG. 3. Immunofluorescence microscopy of *S. cerevisiae*. Cells were grown for 18 h in YPD, YPE, YPG, and YPOB or induced for 18 h in YPOT, harvested, and converted to spheroplasts. Immunofluorescence was carried out with rabbit antiserum against thiolase from *S. cerevisiae*, followed by FITC-conjugated anti-rabbit immunoglobulin G. (A) Epifluorescence microscopy; (B) phase-contrast images of the cells shown in panels A. Bar = 10 μm.

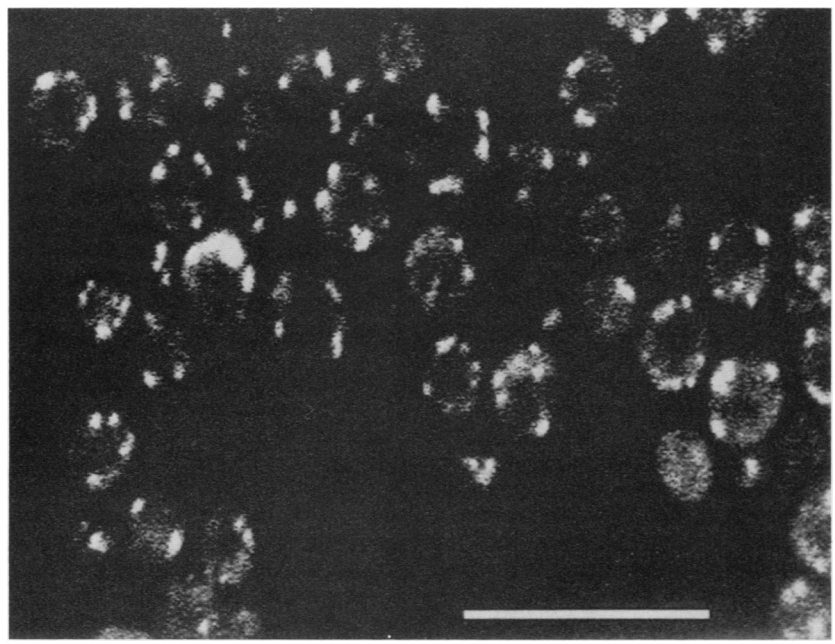

FIG. 4. Confocal microscopy of *S. cerevisiae* grown on YPOB. Cells were grown for 18 h on YPOB, and immunofluorescence with antithiolase was carried out as described in the legend to Fig. 3. Bar = 10 μm.

tory were used successfully for the purification of peroxisomes from *C. tropicalis* (38) did not work for *S. cerevisiae* peroxisomes. In particular, the purified peroxisomes were extremely fragile, and many broke, releasing their content, when we tried to concentrate them by dilution and recentrifugation (Table 2A), which is a prerequisite of the import assays. Moreover, under some gradient conditions, the separation of peroxisomes from mitochondria was inadequate (Table 3, experiment 3 and to a lesser extent experiment 2). Therefore, we tried to isolate the organelles at pH 6 instead of pH 7.2, which had proven useful for *Candida boidinii* according to Goodman et al. (16) and was recently reported to be useful for *S. cerevisiae* (27). Lowering the pH indeed proved very useful for *S. cerevisiae*: some decrease in leakage of peroxisome enzymes was apparent already when crude organelle pellets were recentrifuged (Table 2). A dramatic increase in the stability of peroxisomes was seen when peroxisomes were purified in gradients buffered at pH 6 rather than the higher pH: when the purified peroxisomes were diluted and recentrifuged, approximately 40% of the catalase resedimented if the peroxisomes had been isolated at pH 7.2, whereas ~80% of the catalase resedimented when the purification was done at pH 6 (Table 2).

We tried adding sucrose throughout the Nycodenz gradients at a constant concentration to provide some additional osmotic protection for the peroxisome. This proved useful for catalase at pH 7.2 (Table 2A).

We attempted to increase the yield of peroxisomes by shifting *S. cerevisiae* at high cell density into oleate-containing medium. The yield of peroxisome enzymes may have increased modestly (Table 1B versus 1A). More importantly, we found that the peroxisomes thus induced were significantly more stable, as determined by an assay of the latency of catalase (Table 4).

Many equilibrium density centrifugations were carried out to determine how to optimize the separation of peroxisomes and mitochondria (Table 3). Lowering the pH from 7.5 or 7.2 to 6.0 caused the density of mitochondria to decrease slightly and caused the density of peroxisomes to increase noticeably, resulting in a substantial improvement in the separation of these organelles. This effect was seen both with sucrose gradients and with Nycodenz gradients (Table 3). At pH 6, the absolute peak separation was 0.02 to 0.03 g/cm^3 in sucrose gradients and 0.06 g/cm^3 in Nycodenz gradients containing 0.25 M sucrose. The widths of the mitochondrial and the peroxisomal peaks were substantially less in Nycodenz gradients at pH 6 than in sucrose gradients at the same pH. For these reasons, we chose the following optimal conditions for future peroxisome purifications: induction of peroxisomes in YPOT, use of pH 6 buffers throughout cell fractionation, and use of 15 to 36% Nycodenz gradients containing a constant concentration of 0.25 M sucrose throughout the gradients. The results under these conditions are shown in Tables 5 and 6 and Fig. 6.

Whereas the majority of the cytochrome oxidase (mitochondrial inner membrane) and acyl-CoA oxidase (peroxisomes) activities were recovered in the crude organelle fraction (Table 5, 25,000 × *g* pellet), most of the catalase activity was found in the 25,000 × *g* supernatant fraction. Immunoblotting (Fig. 7, lane 2) demonstrated that this was, at least to a large extent, due to the presence of (cytosolic) catalase T. Alkaline phosphatase, which has been reported to be a vacuolar marker enzyme (42) at least in part (48), and NADPH-cytochrome *c* oxidoreductase, a microsomal marker, were almost exclusively found in the 25,000 × *g* supernatant fraction. When the crude organelles were further fractionated by equilibrium density centrifugation, the peroxisomes were well separated from the other organelles (Fig. 6; compare the distributions of catalase and acyl-CoA oxidase with those of the other marker enzymes). The catalase activity in peroxisomes was identified as catalase A by immunoblotting (Fig. 7, lane 6). The overall yield of catalase A in the peroxisome fraction was unexpectedly high (see legend to Fig. 7).

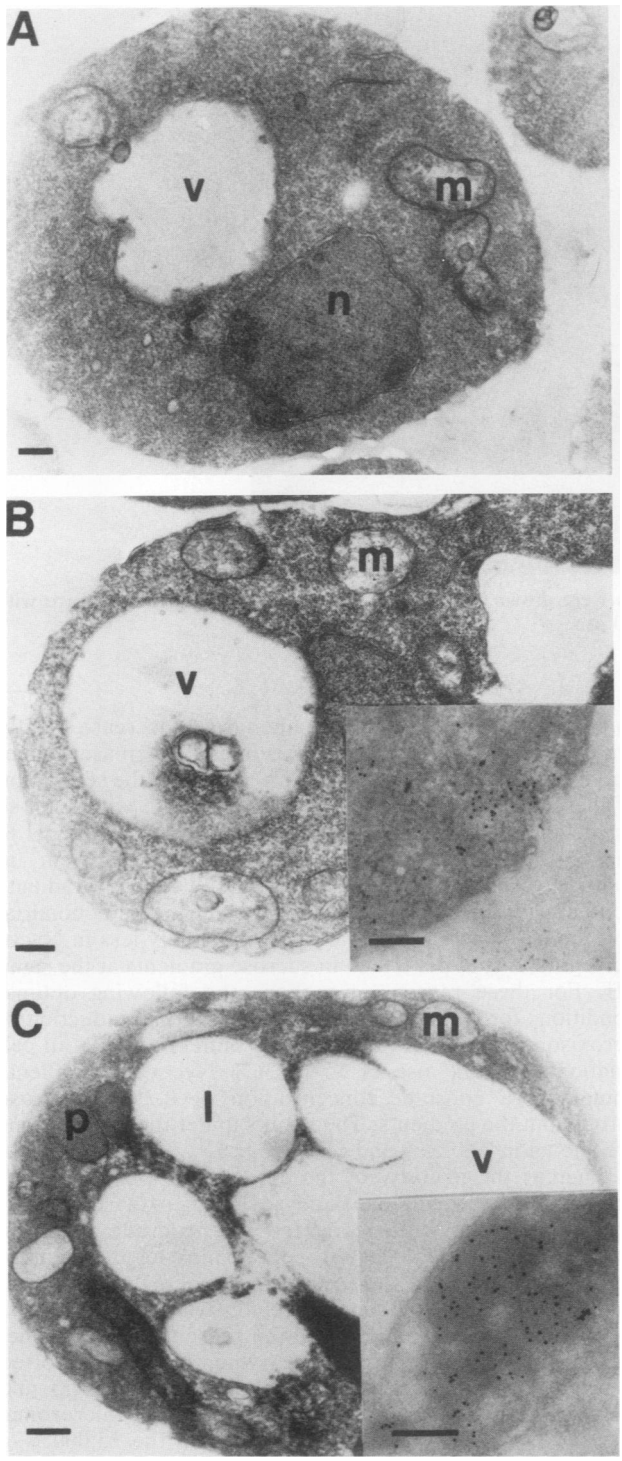

FIG. 5. Electron microscopy of thin sections of *S. cerevisiae* after growth in YPD (A), YPG (B), and YPOB (C). Inserts in panels B and C show immunoelectron microscopy carried out with a cross-reacting rabbit antiserum raised against total peroxisomal protein from *C. tropicalis* followed by protein A-gold. p, Peroxisomes; m, mitochondria; l, lipid droplets; v, vacuole; n, nucleus. Bars = 0.2 μm.

Acyl-CoA oxidase in the peak gradient fraction was purified 31-fold compared with the homogenate (Table 6). Enrichment of catalase activity was 23-fold in the peak peroxisomal fraction. This lesser purification was likely due to the existence of a cytosolic as well as a peroxisomal catalase. The electron microscopic appearance of the peak peroxisomal fraction is shown in Fig. 8. After purification, fixation, and embedding, the peroxisomes had lost a great deal of their contents but were identified unambiguously by immunolabeling. The principle contaminant is damaged mitochondria. The contamination of the peroxisomal fraction with mitochondria may have been underestimated biochemically because the recoveries of cytochrome oxidase in the crude organelle fraction (Table 5) and in the Nycodenz gradient (Fig. 6) were low.

Isocitrate lyase, one of the two enzymes of the bypass reactions of the glyoxylate cycle, is localized in microbodies in other yeasts and fungi (45). However, isocitrate lyase in *S. cerevisiae* was not appreciably associated with the 25,000 × *g* pellet fraction under our fractionation conditions. The 0.4% residual activity in the pellet fraction did not cosediment with the peroxisomes when subjected to density gradient centrifugation (Fig. 6).

In vitro import of catalase A. The *CTA1* gene was expressed in vitro and produced a polypeptide with an apparent molecular mass of 58 kDa (Fig. 9A), which is in good agreement with the expected mass of 58.5 kDa for the catalase A subunit (8). The minor additional bands at 47, 38, 36, 35, and 26 kDa most likely represent translation products of catalase A mRNA that started at internal ATG codons. This mRNA contains seven internal ATGs, which, if all used, would produce proteins with expected masses of 47, 38, 36, 34, 26, 14, and 3 kDa. The band at 29 kDa seems to be a proteolytic degradation product of catalase A, because we observed the formation of a fragment of the same size in the protease protection assay (not shown).

The ^{35}S-labeled catalase A was tested for in vitro import with purified, concentrated peroxisomes. Association with peroxisomes occurred in a time- and temperature-dependent fashion (Fig. 9B). Under the experimental conditions chosen, 62% of catalase A became associated with peroxisomes after 2 h of incubation. One-quarter of this was imported into the organelle in a protease-resistant fashion (Fig. 10). The five smaller bands mentioned above also appear to be imported in a protease-resistant fashion. In several control experiments, ^{35}S-labeled catalase A was completely digested in the supernatants by protease (Fig. 9B), and all catalase was digested in the presence of detergent. When the import assay was performed at 0°C, only 18% of catalase A became associated with peroxisomes after 2 h. Less than 4% of the protein was protease protected, and this residual protease protection did not occur in a time-dependent fashion (Fig. 10). In other control experiments, the import assay was carried out in the absence of organelles (mock) or with mitochondria in place of peroxisomes. After 1 min of incubation, there appeared to be about 10% of catalase A that associated with the organelles in a nonspecific fashion, and about 4% that was protease resistant. This latter figure is similar to the result obtained when translation products were analyzed by rate zonal sedimentation: most sedimented at a rate expected for a catalase A monomer, and 3 to 7% sedimented as much larger aggregates (not shown). We suspect that these aggregates may be protease resistant and stick to membranes. There appeared to be no significant time-dependent change with these controls, except perhaps for a small increase in catalase A associated with mitochon-

TABLE 2. Stability of purified peroxisomes estimated by dilution and resedimentation[a]

Expt	Enzyme activity in pellet (% of activity before centrifugation)	Recovery in pellet + supernatant	
		Amt	%
A. pH 7.2			
Crude organelle fraction			
Catalase	60	5.19 mU	98
Acyl-CoA oxidase	69	3.55 U	107
Nycodenz gradient: peroxisomal peak fraction			
Catalase	13	1.52 mU	106
Acyl-CoA oxidase	41	0.43 U	95
Nycodenz–0.25 M sucrose gradient: peroxisomal peak fraction			
Catalase	40	1.86 mU	101
Acyl-CoA oxidase	43	0.75 U	108
B. pH 6.0			
Crude organelle fraction			
Catalase	78	12.5 mU	115
Acyl-CoA oxidase	84	7.16 U	103
Sucrose gradient: peroxisomal peak fraction			
Catalase	77	2.11 mU	116
Acyl-CoA oxidase	86	0.80 U	103
Nycodenz–0.25 M sucrose gradient: peroxisomal peak fraction			
Catalase	79	2.45 mU	117
Acyl-CoA oxidase	61	1.62 U	109

[a] The fractions were obtained from cells grown in YPOB as described in Materials and Methods. Aliquots of the fractions were diluted fivefold with 0.5 M sucrose in either 2.5 mM MOPS (pH 7.2)–1 mM EDTA (A) or 5 mM MES (pH 6.0)–1mM EDTA (B) and centrifuged at 16,000 × *g* for 15 min in a microcentrifuge.

dria at 2 h at 26°C. We cannot be certain whether this slight increase is due to minor peroxisomal contamination or a slight infidelity in the assay.

DISCUSSION

The results presented above confirm the report of Veenhuis et al. (46) that peroxisomes can be induced to proliferate in *S. cerevisiae* by growth of the organism on oleic acid. By trial and error, we have succeeded in finding conditions in which peroxisomes can be isolated in sufficient quantity and adequate stability to allow in vitro assays of protein import into the organelle, as we have done with *C. tropicalis* peroxisomes (38). This now makes it possible to combine the advantages of in vitro protein import studies with the advantages of molecular genetics that are highly developed for *S. cerevisiae*.

The principal methodological improvements were to change the growth conditions, lower the pH to 6 during the isolation procedures, and stabilize the peroxisomes osmotically. Before these improvements, most of the catalase leaked out of the peroxisomes during the isolation procedures, although another enzyme, acyl-CoA oxidase, did not leak out appreciably. This differential enzyme leakage has been seen previously with deliberately damaged rat liver peroxisomes (1). Under the final optimized conditions, the purified *S. cerevisiae* peroxisomes retained both enzymes to a similar extent (61 to 79%; Table 2).

Despite these improvements and the biochemical evidence for stability, the purified peroxisomes looked rather empty

TABLE 3. Densities of mitochondria and peroxisomes after isopycnic centrifugation in various media

Expt	Growth medium	Density gradient	pH	Density (g/ml)[a]		
				Mitochondria, cytochrome oxidase	Peroxisomes	
					Catalase	Acyl-CoA oxidase
1	YPOB	Nycodenz	7.5	1.093	1.169	1.161
2	YPOB	Nycodenz–0.25 M sucrose	7.5	1.135	1.158	1.159
3	YPOB	Sucrose	7.2	1.181	1.192	1.192
4	YPOB	Sucrose	6.0	1.178	1.198	1.197
5	YPOB	Nycodenz–0.25 M sucrose	6.0	1.126	1.185	1.183
6	YPOT	Sucrose	6.0	1.168	1.199	1.199
7	YPOT	Nycodenz–0.25 M sucrose	6.0	1.103	1.167	1.168

[a] Peak fraction determined by enzymatic analysis of the marker enzymes. The 25,000 × *g* pellet fractions from YPOB-grown or YPOT-induced cells were subjected to density gradient centrifugation as described in Materials and Methods. The densities of the Nycodenz gradients containing 0.25 M sucrose were corrected for the refractive index contributed by sucrose.

TABLE 4. Catalase latency of peroxisomes isolated at pH 6.0

Fraction[a]	Catalase latency[b]			
	YPOB-grown cells		YPOT-induced cells	
	0.25 M	0.50 M	0.25 M	0.50 M
Crude mitochondrial fraction	9	6	25	57
Peroxisomal peak fraction from:				
Sucrose gradient	ND	13	ND	48
Nycodenz–0.25 M sucrose gradient	ND	11	45	58
Concentrated peroxisomes[c]	ND	ND	ND	72

[a] The fractions were diluted 20-fold in 5 mM MES (pH 6.0)–1 mM EDTA containing 0.25 or 0.50 M sucrose, as indicated.

[b] Determined at 0°C and expressed as [(total activity − free activity)/total activity] × 100. Total activity is the catalase activity determined in 1.1 ml of assay medium in the presence of 0.1% (vol/vol) Triton X-100. Free activity is the catalase activity determined under the same assay conditions in the absence of Triton X-100. ND, Not determined.

[c] Obtained from a peroxisomal peak fraction of a Nycodenz–0.25 M sucrose gradient as described in Materials and Methods.

TABLE 6. Purification of peroxisomes, optimized procedure

Fraction	Catalase		Acyl-CoA oxidase	
	Sp act (mU/mg)	Purification[a]	Sp act (U/mg)	Purification
Homogenate	12.6	1	1.2	1
1,500 × *g* pellet	6.2	0.49	0.9	0.75
Postnuclear supernatant	22.3	1.77	3.1	2.58
25,000 × *g* supernatant	17.5	1.39	1.3	1.08
25,000 × *g* pellet	39.2	3.11	8.6	7.17
Peroxisomal peak fraction (Nycodenz–0.25 M sucrose)	291.8	23.2	37.2	31.0

[a] Ratio of the fraction's specific activity to the homogenate's specific activity.

when examined by electron microscopy. We think that this must be due in part to a loss of content during the preparation of the samples for microscopy, because clearly the soluble enzymes sediment with the membranes and are latent. We have tried a variety of alternative fixation procedures, thus far without any improvement in retaining the organelles' content. It is possible that fixation is affected by growth on oleic acid, which may have altered the composition of the membrane phospholipids. In any case, loss of content seems not to impair the ability of the peroxisomal membranes to import proteins in vitro: rat liver peroxisomes that had lost much of their contents functioned well for import (22).

The most useful tool for assessing peroxisome proliferation proved to be immunofluorescence microscopy. Under many growth conditions, peroxisomes are so small and rare as to be absent altogether from most random thin sections prepared for electron microscopy. We report here for the first time the successful visualization of *S. cerevisiae* peroxisomes by immunofluorescence. By focusing up and down, it was possible to find at least one small peroxisome, and generally three to six, in every cell. This observation is consistent with our understanding that new peroxisomes form by division from preexisting peroxisomes and therefore that every cell must contain at least one peroxisome if peroxisomes are to be induced in that cell or its progeny (5, 24, 25).

There was a good correlation between the extent of induction of acyl-CoA oxidase activity (a key marker enzyme for fatty acid-induced peroxisomes) measured in cell extracts and the immunofluorescence impression of peroxisome abundance. It is noteworthy that the extent of peroxisome proliferation is consistently less in our experience with *S. cerevisiae* than it is with *C. tropicalis*. Although occasional electron micrographic cross sections show an abundance of large peroxisomes in *S. cerevisiae* grown on oleate (10, 46), our typical random sections (Fig. 5) have fewer, smaller peroxisomes than do *C. tropicalis* similarly grown on oleate (13, 38). In any case, none of the yeast species grown on oleic acid show the amazing peroxisome-filled cytoplasms that have been seen in certain methanotrophic yeasts grown in methanol chemostats (47).

Catalase is the prototypical marker enzyme for peroxisomes, identifying the organelle in animals, plants, and a variety of unicellular organisms (5, 25). The existence of catalase-containing peroxisomes in *S. cerevisiae* was first reported by Avers and Federman (2). Subsequently, it was found that this yeast contains two catalase isozymes: an atypical organelle-associated catalase A and a typical soluble catalase T (33, 34). The catalase A was later reported to be associated with the vacuole, not with peroxisomes (43). By immunoblotting of our subcellular fractions with isozyme-specific antibodies, we demonstrated unequivocally that catalase A is a peroxisomal enzyme and confirmed the nonsedimentability of catalase T. The association of catalase A with peroxisomes was independently shown by Skoneczny et al., who used a catalase T-deficient mutant (35).

We assayed one of the key glyoxylate cycle enzymes,

TABLE 5. Differential centrifugation, optimized procedure[a]

Enzyme	Homogenate	Postnuclear supernatant	25,000 × *g* supernatant		25,000 × *g* pellet		Recovery (%)
			Amt	Recovery (%)	Amt	Recovery (%)	
Protein	1,450 mg	483 mg	455 mg	94	84.2 mg	17	111
Cytochrome oxidase	1.68 U	1.36 U	0.08 U	6	0.59 U	43	49
Catalase	13.3 U	9.94 U	8.41 U	85	3.51 U	35	120
Acyl-CoA oxidase	2,250 U	1,350 U	422 U	31	823 U	61	92
Isocitrate lyase	4.22 U	2.82 U	2.23 U	79	0.011 U	0.4	79.4
Alkaline phosphatase	8.95 U	1.33 U	1.18 U	88	0.023 U	2	90
Cytochrome *c* reductase	3.53 U	0.55 U	0.98 U	178	0.072 U	13	191

[a] Cells induced in 500 ml of YPOT medium were fractionated by differential centrifugation as described in Materials and Methods. Percentage recoveries refer to the postnuclear supernatant fraction.

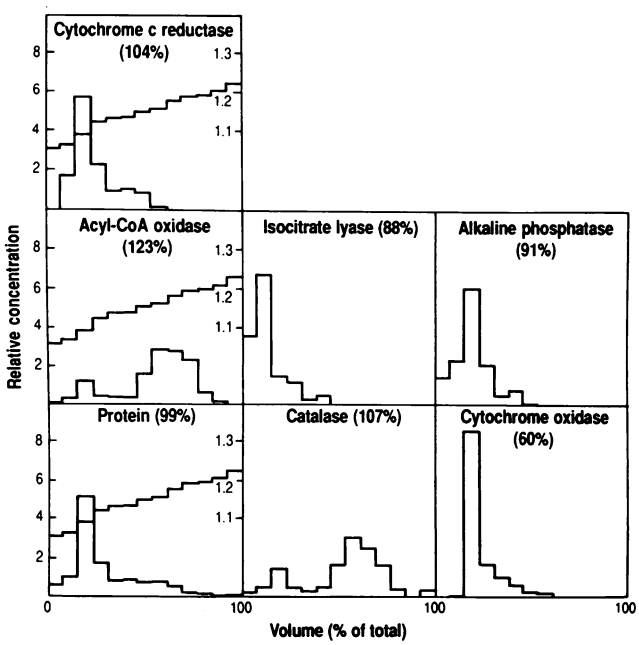

FIG. 6. Density gradient centrifugation in Nycodenz, optimized procedure. A crude organelle fraction from oleate-induced *S. cerevisiae* (Table 5, 25,000 × *g* pellet), containing 80 mg of protein in 2.8 ml, was layered on top of a linear 15 to 36% (wt/vol) Nycodenz gradient in 5 mM MES (pH 6.0)–1 mM EDTA–0.1% (vol/vol) ethanol–0.25 M sucrose. Centrifugation was for 1 h at 35,000 rpm in a Sorvall Vt865B vertical rotor. Distributions of protein and marker enzymes are plotted according to Leighton et al. (26). Staircases indicate the densities of the fractions; recoveries are given in percent.

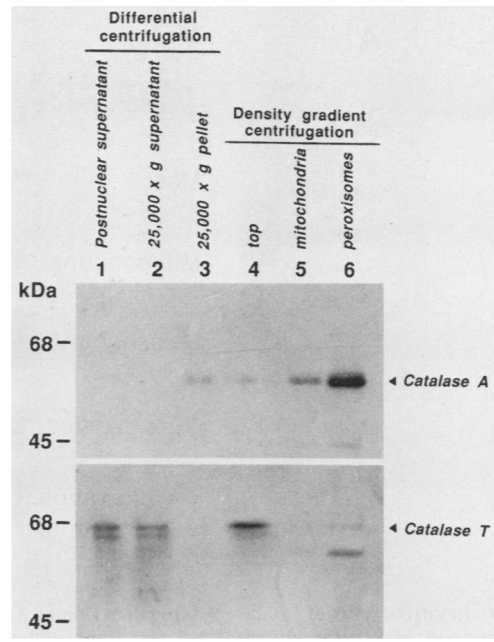

FIG. 7. Immunoblot of catalase A (top) and catalase T (bottom) in subcellular fractions obtained during organelle isolation. Lanes: 1, postnuclear supernatant; 2, 25,000 × *g* supernatant fraction from differential centrifugation; 3, 25,000 × *g* pellet fraction from differential centrifugation; 4 to 6, fractions of a 30 to 60% (wt/wt) sucrose density gradient (pH 6.0) (lanes 4, top fraction; lanes 5, mitochondrial peak fraction; lanes 6, peroxisomal peak fraction). To evaluate the relative distributions of the two catalases (A and T), we tried to load in each lane the same total amount of catalase enzymatic activity (5.3 mU) rather than the same amount of protein. The anti-catalase T cross-reacts weakly with catalase A. The unexpected strong signal with anti-catalase A in lanes 6 is unexplained.

isocitrate lyase, that is found only in glyoxysomes in germinating plants (45), in fungi (14, 23, 45), and in *Tetrahymena pyriformis* (30). This enzyme was entirely nonsedimentable in our experiments. This may mean that isocitrate lyase is located in the cytosol of *S. cerevisiae*, which would be an unusual intracellular location. Alternatively, it could be that the enzyme leaked out of the peroxisomes despite our best efforts to maintain the integrity of the organelles. Szabo and Avers (44) reported that some isocitrate lyase was present in *S. cerevisiae* peroxisomes. The other original observations by Avers and co-workers (2, 44) about *S. cerevisiae* peroxisomes have now all been confirmed, and we take seriously the possibility of leakage.

In summary, it has been possible to isolate peroxisomes from *S. cerevisiae* that have a good stability as judged by catalase latency (60 to 70%; Table 4) and the ability to be recentrifuged without appreciable loss of enzyme activities. Morphologically, they appear rather empty. These concentrated peroxisomes have been used for in vitro protein import studies with catalase A, which was expressed in vitro from the *CTA1* gene. Association of catalase A with peroxisomes appeared to be a very rapid process. After 1 min of incubation, 23% association was observed. It seems reasonable to conclude that this reflects the initial binding events of catalase to unoccupied binding sites (receptors) on the peroxisomal membrane. Further association of catalase with peroxisomes occurred between 1 and 120 min of incubation. Import of catalase A into peroxisomes also occurred in a time-dependent fashion: after 120 min, 16% of the catalase was resistant to protease treatment. The import efficiency of

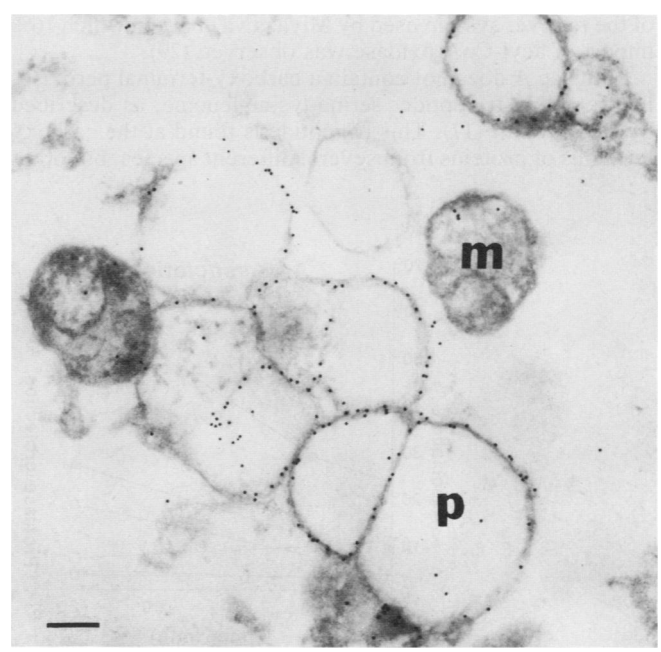

FIG. 8. Immunoelectron microscopy of the peak peroxisome fraction. Cross-reacting rabbit antiserum raised against total peroxisomal protein of *C. tropicalis* was used, followed by protein A-gold. p, Peroxisomes; m, mitochondria. Bar = 0.2 μm.

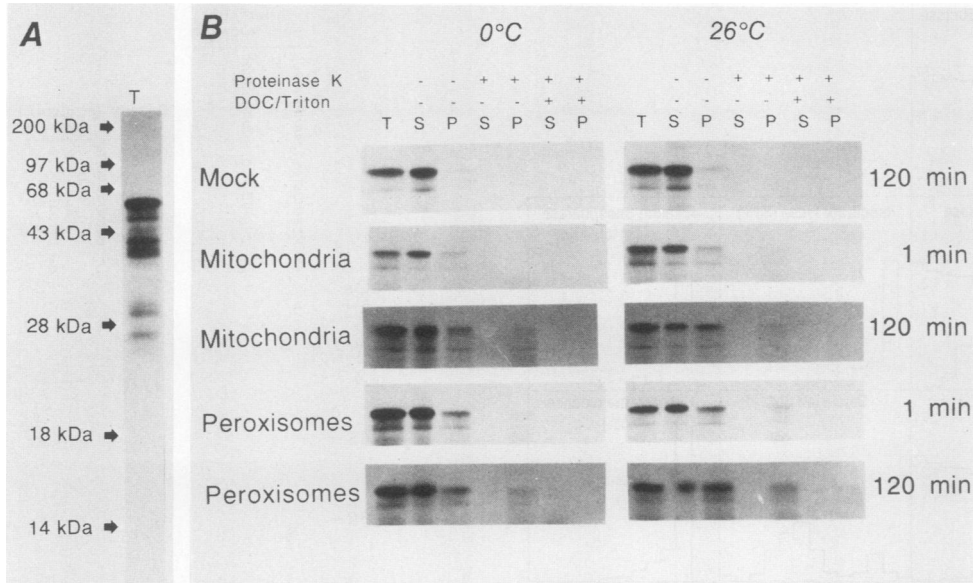

FIG. 9. In vitro import of catalase A into peroxisomes. (A) In vitro expression of *CTA1*. A pGEM4 plasmid containing the catalase A gene was transcribed, and the resulting mRNA was translated in wheat germ extract. Translation product (T) was incubated with concentrated peroxisomes, and 2 μl of this mixture was analyzed by SDS-PAGE and fluorography. Positions of molecular mass standards (myosin, phosphorylase *b*, bovine serum albumin, ovalbumin, α-chymotrypsinogen, β-lactoglobulin, and lysozyme) are indicated. (B) Assay in which newly synthesized catalase A in wheat germ extract was mixed with peroxisomes or mitochondria or SEM buffer alone (mock) and incubated at 0 or 26°C for the times indicated. Three equal samples of each were (+) or were not (−) treated with 5 μg of proteinase K at 0°C for 30 min in the presence (+) or absence (−) of detergent. Supernatants (S) and pellets (P) were then separated by centrifugation, and equal amounts of each were analyzed by SDS-PAGE and fluorography.

catalase is considerably higher than that of full-length acyl-CoA oxidase (~3%) seen by Small et al. in the *C. tropicalis* import system (39), and lower than the 30% import of acyl-CoA oxidase in the rat liver import system as described by Imanaka et al. (22). It is comparable to the import efficiency of the rat liver system used by Miyazawa et al., in which 16% import of acyl-CoA oxidase was observed (29).

Catalase A does not contain a carboxy-terminal peroxisomal targeting tripeptide, serine-lysine-leucine, as described by Gould et al. (17). This tripeptide is found at the carboxy terminus of proteins from several different species, but other peroxisomal proteins lack this feature (5, 24). The three carboxy-terminal amino acids of catalase A are serine-lysine-phenylalanine (8). This tripeptide has been found to be nonfunctional for peroxisomal targeting in CV-1 monkey kidney cells in vivo (17). A serine-lysine-leucine tripeptide is located at positions 2 to 4 of the predicted catalase amino acid sequence. In our in vitro import experiment, the minor translation products with masses of 26 to 47 kDa appeared to be imported into peroxisomes with efficiencies similar to that of full-length, 58.5-kDa catalase A (not shown). Since these smaller peptides appear to arise by initiation of translation at

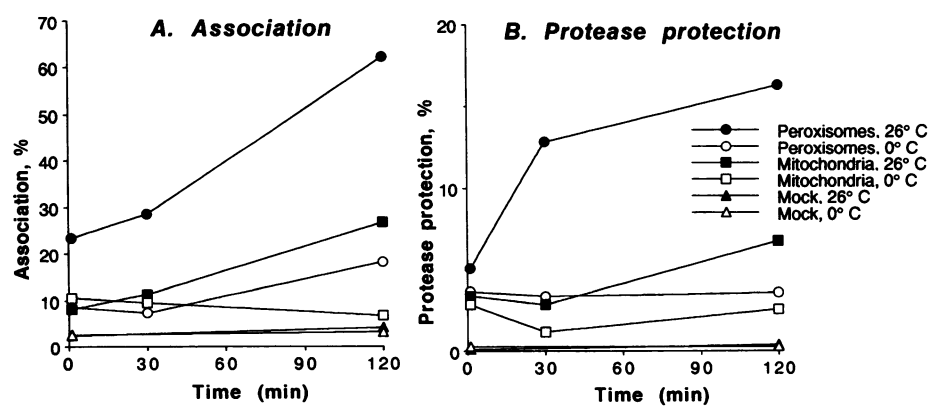

FIG. 10. Quantitation of ^{35}S-labeled catalase A in the pellet fractions of the experiment of Fig. 9. Radioactivity was determined by densitometric scanning of the fluorograms. Association is the amount of ^{35}S-labeled catalase found in the pellet fractions after centrifugation without protease treatment. Protease protection is the amount of radioactive catalase found in the pellet fractions after treatment with proteinase K. Results are expressed as a percentage of the ^{35}S-labeled catalase A added to the import assay.

internal ATGs, the results suggest that there is topogenic information in the carboxy-terminal half of catalase A. It should be feasible now to investigate catalase A topogenesis both with the in vitro import assay described here and by immunofluorescence after expression in vivo.

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REFERENCES

1. **Alexson, S. E. H., Y. Fujiki, H. Shio, and P. B. Lazarow.** 1985. Partial disassembly of peroxisomes. J. Cell Biol. **101**:294–305.
2. **Avers, C. J., and M. Federman.** 1968. The occurrence in yeast of cytoplasmic granules which resemble microbodies. J. Cell Biol. **37**:555–559.
3. **Baudhuin, P., H. Beaufay, Y. Rahman-Li, O. Z. Sellinger, R. Wattiaux, P. Jacques, and C. de Duve.** 1964. Tissue fractionation studies. 17. Intracellular distribution of monoamine oxidase, aspartate aminotransferase, alanine aminotransferase, D-amino acid oxidase, and catalase in rat-liver tissue. Biochem. J. **92**:179–184.
4. **Beaufay, H., A. Amar-Costesec, E. Feytmans, D. Thinès-Sempoux, M. Wibo, M. Robbi, and J. Berthet.** 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. I. Biochemical methods. J. Cell Biol. **61**:188–200.
5. **Borst, P.** 1989. Peroxisome biogenesis revisited. Biochem. Biophys. Acta **1008**:1–13.
6. **Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
7. **Cohen, G., F. Fessl, A. Traczyk, J. Rytka, and H. Ruis.** 1985. Isolation of the catalase A gene of *Saccharomyces cerevisiae* by complementation of the ctal mutation. Mol. Gen. Genet. **200**: 74–79.
8. **Cohen, G., W. Rapatz, and H. Ruis.** 1988. Sequence of the *Saccharomyces cerevisiae* CTA 1 gene and amino acid sequence of catalase A derived from it. Eur. J. Biochem. **176**:159–163.
9. **Dixon, G. H., and H. L. Kornberg.** 1959. Assay methods for key enzymes of the glyoxylate cycle. Biochem. J. **72**:3P.
10. **Erdmann, R., M. Veenhuis, D. Mertens, and W.-H. Kunau.** 1989. Isolation of peroxisome-deficient mutants of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **86**:5419–5423.
11. **Erickson, A. H., and G. Blobel.** 1983. Cell-free translation of messenger RNA in a wheat germ system. Methods Enzymol. **96**:38–50.
12. **Fujiki, Y., R. A. Rachubinski, R. M. Mortensen, and P. B. Lazarow.** 1985. Synthesis of 3-ketoacyl-CoA thiolase of rat liver peroxisomes on free polyribosomes as a larger precursor. Induction of thiolase mRNA activity by clofibrate. Biochem. J. **226**:697–704.
13. **Fujiki, Y., R. A. Rachubinski, A. Zentella-Dehesa, and P. B. Lazarow.** 1986. Induction, identification and cell-free translation of mRNAs coding for peroxisomal proteins in *Candida tropicalis*. J. Biol. Chem. **261**:15787–15793.
14. **Fukui, S., and A. Tanaka.** 1979. Peroxisomes of alkane- and methanol-grown yeasts. J. Appl. Biochem. **1**:171–201.
15. **Garoff, H.** 1985. Using recombinant DNA techniques to study protein targeting in the eucaryotic cell. Annu. Rev. Cell Biol. **1**:403–445.
16. **Goodman, J. M., C. W. Scott, P. N. Donahue, and J. P. Atherton.** 1984. Alcohol oxidase assembles post-translationally into the peroxisomes of *Candida boidinii*. J. Biol. Chem. **259**:8485–8493.
17. **Gould, S. J., G.-A. Keller, N. Hosken, J. Wilkinson, and S Subramani.** 1989. A conserved tripeptide sorts proteins to peroxisomes. J. Cell Biol. **108**:1657–1664.
18. **Gould, S. J., G.-A. Keller, M. Schneider, S. H. Howell, L. J. Garrard, J. M. Goodman, B. Distel, H. Tabak, and S. Subramani.** 1990. Peroxisomal protein import is conserved between yeast, plants, insects and mammals. EMBO J. **9**:85–90.
19. **Hansen, H., and R. Roggenkamp.** 1989. Functional complementation of catalase-defective peroxisomes in a methylotropic yeast by import of catalase A from *Saccharomyces cerevisiae*. Eur. J. Biochem. **184**:173–179.

19a. **Hartig, A., M. Ogris, G. Cohen, and M. Binder.** 1990. Fate of highly expressed proteins destined to peroxisomes in *Saccharomyces cerevisiae*. Curr. Genet. **18**:23–27.

20. **Hartig, A., and H. Ruis.** 1986. Nucleotide sequence of the *Saccharomyces cerevisiae* CTT1 gene and deduced amino-acid sequence of yeast catalase T. Eur. J. Biochem. **160**:487–490.
21. **Hörtner, H., G. Ammerer, E. Hartter, B. Hamilton, J. Rytka, T. Bilinski, and H. Ruis.** 1982. Regulation of synthesis of catalases and iso-1-cytochrome c in *Saccharomyces cerevisiae* by glucose, oxygen and heme. Eur. J. Biochem. **128**:179–184
22. **Imanaka, T., G. M. Small, and P. B. Lazarow.** 1987. Translocation of acyl-CoA oxidase into peroxisomes requires ATP hydrolysis but not a membrane potential. J. Cell Biol. **105**:2915–2922.
23. **Kionka, C., and W.-H. Kunau.** 1985. Inducible β-oxidation pathway in *Neurospora crassa*. J. Bacteriol. **161**:153–157.

23a. **Kunau, W. H.** Personal communication.

24. **Lazarow, P. B.** 1989. Peroxisome biogenesis. Curr. Opin. Cell Biol. **1**:630–634.
25. **Lazarow, P. B., and Y. Fujiki.** 1985. Biogenesis of peroxisomes. Annu. Rev. Cell Biol. **1**:489–530.
26. **Leighton, F., B. Poole, H. Beaufay, P. Baudhuin, J. W. Coffey, S. Fowler, and C. de Duve.** 1968. The large scale separation of peroxisomes, mitochondria, and lysosomes from the livers of rats injected with Triton WR-1339. J. Cell Biol. **37**:482–513.
27. **Lewin, A. S., V. Hines, and G. M. Small.** 1990. Association of glyoxylate and beta-oxidation enzymes with peroxisomes of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10**:1399–1405.
28. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
29. **Miyazawa, S., T. Osumi, T. Hashimoto, K. Ohno, S. Miura, and Y. Fujiki.** 1989. Peroxisome targeting signal of rat liver acyl coenzyme A oxidase resides at the carboxy terminus. Mol. Cell. Biol. **9**:83–91.
30. **Müller, M., J. F. Hogg, and C. de Duve.** 1968. Distribution of tricarboxylic acid cycle enzymes and glyoxylate cycle enzymes between mitochondria and peroxisomes in *Tetrahymena pyriformis*. J. Biol. Chem. **243**:5385–5395.
31. **Santos, M. J., T. Imanaka, H. Shio, and P. B. Lazarow.** 1988. Peroxisomal integral membrane proteins in control and Zellweger fibroblasts. J. Biol. Chem. **263**:10502–10509.
32. **Schekman, R.** 1985. Protein localization and membrane traffic in yeast. Annu. Rev. Cell Biol. **1**:115–143.
33. **Seah, T. C. M., A. R. Bhatti, and J. G. Kaplan.** 1973. Novel catalatic proteins of bakers' yeast. I. An atypical catalase. Can. J. Biochem. **51**:1551–1555.
34. **Seah, T. C. M., and J. G. Kaplan.** 1973. Purification and properties of the catalase of bakers' yeast. J. Biol. Chem. **248**:2889–2893.
35. **Skoneczny, M., A. Chelstowska, and J. Rytka.** 1988. Study of the coinduction by fatty acids of catalase A and acyl-CoA oxidase in standard and mutant *Saccharomyces cerevisiae* strains. Eur. J. Biochem. **174**:297–302.
36. **Small, G. M., K. Burdett, and M. J. Connock.** 1985. A sensitive spectrophotometric assay for peroxisomal acyl-CoA oxidase. Biochem. J. **227**:205–210.
37. **Small, G. M., T. Imanaka, and P. B. Lazarow.** 1988. Immunoblotting of hydrophobic integral membrane proteins. Anal. Biochem. **169**:405–409.
38. **Small, G. M., T. Imanaka, H. Shio, and P. B. Lazarow.** 1987. Efficient association of in vitro translation products with purified, stable *Candida tropicalis* peroxisomes. Mol. Cell. Biol.

7:1848–1855.
39. **Small, G. M., L. J. Szabo, and P. B. Lazarow.** 1988. Acyl-CoA oxidase contains two targeting sequences each of which can mediate protein import into peroxisomes. EMBO J. **7**:1167–1173.
40. **Spevak, W., F. Fessl, J. Rytka, A. Traczyk, M. Skoneczny, and H. Ruis.** 1983. Isolation of catalase T structural gene of *Saccharomyces cerevisiae* by functional complementation. Mol. Cell. Biol. **3**:1545–1551.
41. **Strathern, J. N., E. W. Jones, and J. R. Broach (ed.).** 1981. The molecular biology of the yeast *Saccharomyces*: life cycle and inheritance. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
42. **Strathern, J. N., E. W. Jones, and J. R. Broach (ed.).** 1982. The molecular biology of the yeast *Saccharomyces*: metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
43. **Susani, M., P. Zimniak, F. Fessl, and H. Ruis.** 1976. Localization of catalase A in vacuoles of *Saccharomyces cerevisiae*: evidence for the vacuolar nature of isolated "yeast peroxisomes." Hoppe-Seyler's Z. Physiol. Chem. **357**:961–970.
44. **Szabo, A. S., and C. J. Avers.** 1969. Some aspects of regulation of peroxisomes and mitochondria in yeast. Ann. N.Y. Acad. Sci. **168**:302–312.
45. **Tolbert, N. E.** 1981. Metabolic pathways in peroxisomes and glyoxysomes. Annu. Rev. Biochem. **50**:133–157.
46. **Veenhuis, M., M. Mateblowski, W.-H. Kunau, and W. Harder.** 1987. Proliferation of microbodies in *Saccharomyces cerevisiae*. Yeast **3**:77–84.
47. **Veenhuis, M., J. P. Van Dijken, and W. Harder.** 1976. Cytochemical studies on the localization of methanol oxidase and other oxidases in peroxisomes of methanol-grown *Hansenula polymorpha*. Arch. Microbiol. **111**:123–135.
48. **Wiemken, A., M. Schellenberg, and K. Urech.** 1979. Vacuoles: the sole compartments of digestive enzymes in yeast (*Saccharomyces cerevisiae*)? Arch. Microbiol. **123**:23–35.
49. **Zimniak, P., E. Hartter, W. Woloszczuk, and H. Ruis.** 1976. Catalase biosynthesis in yeast: formation of catalase A and catalase T during oxygen adaptation of *Saccharomyces cerevisiae*. Eur. J. Biochem. **71**:393–398.