Association of Glyoxylate and Beta-Oxidation Enzymes with Peroxisomes of *Saccharomyces cerevisiae*

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Although peroxisomes are difficult to identify in *Saccharomyces cerevisiae* under ordinary growth conditions, they proliferate when cells are cultured on oleic acid. We used this finding to study the protein composition of these organelles in detail. Peroxisomes from oleic acid-grown cells were purified on a discontinuous sucrose gradient; they migrated to the 46 to 50% (wt/wt) sucrose interface. The peroxisomal fraction was identified morphologically and by the presence of all of the enzymes of the peroxisomal beta-oxidation pathway. These organelles also contained a significant but minor fraction of two enzymes of the glyoxylate pathway, malate synthase and malate dehydrogenase-2. The localization of malate synthase in peroxisomes was confirmed by immunoelectron microscopy. It is postulated that glyoxylate pathway enzymes are readily and preferentially released from peroxisomes upon cell lysis, accounting for their incomplete recovery from isolated organelles. Small uninduced peroxisomes from glycerol-grown cultures were detected on sucrose gradients by marker enzymes. Under these conditions, catalase, acyl-coenzyme A oxidase, and malate synthase cofractionated at equilibrium close to the mitochondrial peak, indicating smaller, less dense organelles than those from cells grown on oleic acid. Peroxisomal membranes from oleate cultures were purified by buoyant density centrifugation. Three abundant proteins of 24, 31, and 32 kilodaltons were observed.

Peroxisomes are ubiquitous organelles of eucaryotic cells. Organelle composition shows a high degree of plasticity; levels of metabolic enzymes can vary greatly among cell types, developmental stages, and environmental conditions (20, 60). For example, the function of peroxisomes in plant seedlings is devoted almost exclusively to conversion of triglyceride stores into useful carbohydrate through fatty acid beta-oxidation, generating acetyl-coenzyme A (acetyl-CoA), which is assimilated through the glyoxylate cycle. In contrast, organelles in green leaves function primarily in the oxidative photosynthetic carbon cycle. Important functions of peroxisomes in animal cells include both anabolic and catabolic transformations. Among the known anabolic pathways which utilize peroxisomal enzymes in various tissues are the biosyntheses of lipids, such as plasmalogens and platelet-activating factor; bile acids; and, perhaps, cholesterol. Catabolic functions include conversion of fatty acids to acetyl-CoA and oxidation of both alcohols and amines. Several human diseases have been described which are characterized by absence of peroxisomal function (52); in severe cases, patients die at an early age and have extremely high levels of long-chain fatty acids and other metabolites normally degraded in peroxisomes in their sera. Because of this variability in function, the common properties of this class of organelles are not easily apparent. Most peroxisomal pathways contain flavin oxidase that generate hydrogen peroxide; catalase is present to degrade this reactive oxygen species. Most, if not all, peroxisomes also contain the beta-oxidation pathway for fatty acid utilization.

Peroxisomes (often called by the morphologic term microbodies) of eucaryotic microorganisms are necessary for utilization of many carbon and nitrogen substrates. Conse-quently, the size and number of microbodies can be manipulated by external stimuli. Microbodies can be induced in *Neurospora crassa* by acetate (11), in trypanosomes during the slender phase of the life cycle in the mammalian bloodstream (46), and by methanol, fatty acids, *n*-alkanes, and other growth substrates in yeasts (67; see below). The potential for obtaining abundant organelles from these sources makes microorganisms useful models for the study of peroxisomal structure, function, and assembly.

The previous efforts in our laboratories have concentrated on the peroxisomes of methylotrophic yeasts, particularly *Candida boidinii* and *Hansenula polymorpha*. After growth on methanol as a sole carbon and energy source, peroxisomes become the most abundant organelle, greatly facilitating studies of its composition, function, and assembly (5, 17, 24–26). While the usefulness of these untraditional yeasts has been readily apparent, a genetic approach toward understanding peroxisomal assembly in these yeasts has proven difficult to establish (12). Since classical and molecular genetics have been well developed in *Saccharomyces cerevisiae*, this organism has great potential for the study of peroxisomal assembly and function. However, until recently, evidence for the presence of peroxisomes in *S. cerevisiae* has been scant and controversial. Early reports of the presence of catalase, isocitrate lyase, and malate synthase in peroxisomes (3, 4, 47) were disputed. The glyoxylate cycle enzymes were reported to be soluble (18, 48), while catalase A was reported to be vacuolar (58). The issue of whether peroxisomes exist at all in *S. cerevisiae* was largely resolved recently with the induction of large peroxisomes by oleic acid (68). The presence of catalase in these organelles was demonstrated by cytochemical staining (68). Catalase A was also shown to cofractionate with acyl-CoA oxidase (54) and other enzymes of the beta-oxidation pathway (64). The localization of the glyoxylate cycle enzymes

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has only recently been reassessed (37). Furthermore, the dependence of peroxisomes for growth on fatty acids in *S. cerevisiae* has been useful for the isolation of peroxisomal mutants (19), which may be useful for the study of human disease.

The dramatic proliferation of peroxisomes observed after growth on oleic acid (68) suggests a regulated pathway for organellar induction and permits characterization of the organellar structure and metabolism. By using the biochemical approaches developed with *C. boidinii*, we achieved purification and subfractionation of peroxisomes after oleate induction, as well as initial characterization of the organelle from uninduced cultures. Several enzymes of the glyoxylate pathway were detected in these peroxisomes. We also purified the peroxisomal membrane proteins of *S. cerevisiae* and compared them with the substrate-nonspecific membrane proteins of *C. boidinii* (25; J. M. Goodman, S. B. Trapp, H. Hwang, and M. Veenhuis, J. Cell Sci., in press).

MATERIALS AND METHODS

Cell culture. *S. cerevisiae* MMYO11 (*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* Ole⁺), a derivative of W303-1B (from Alan Myers) which grows well on oleate, and *C. boidinii* ATCC 32195 were used throughout this study. MMYO11 was cultured on YPG (1% yeast extract, 2% peptone, 3% glycerol), YPG plus 0.1% (vol/vol) oleic acid, or a semisynthetic medium containing 0.1% (vol/vol) oleate as previously described (65). *C. boidinii* was cultured on semisynthetic glucose (1%) or semisynthetic oleate (0.1%).

Preparation of peroxisomes. Peroxisomes were prepared from *S. cerevisiae* and *C. boidinii* essentially as described for the latter yeast (26). For MMYO11, overnight cultures were inoculated in semisynthetic oleate from precultures in YPG plus 0.1% glucose. All cultures were harvested at an optical density at 600 nm of 2.5 to 3.5 in a volume of either 10 or 2.5 liters. Cells were treated with 0.1 M Tris sulfate (pH 9.3)–10 mM dithiothreitol before conversion to spheroplasts in 1 M sorbitol–20 mM potassiuim phosphate buffer (pH 7.5) with 1 mg of Zymolyase 100K (Seikagaku) with cells at an optical density at 600 nm of 500 to 600. Spheroplasts were suspended in 1 M SMP (sorbitol at the molarity indicated, 5 mM 4-morpholineethanesulfonic acid–NaOH [pH 5.5], 1 mM phenylmethylsulfonyl fluoride) at 4°C and then osmotically lysed by addition of 1.3 volumes of 0.25 M SMP. Lysis was accelerated by pipetting the spheroplast solution until it was 75 to 90% by microscopic observation. The lysate was osmotically adjusted back to 1 M sorbitol by addition of 1.75 M SMP.

Unlysed cells, large organelles, and other cell debris were removed from the lysate by centrifugation at 600 × *g* for 20 min. The pellet was suspended in 1 M SMP and respun; the resulting supernatant was added to the first. The combined supernatant was centrifuged at 22,000 × *g* (maximum) for 20 min to obtain a crude pellet consisting mainly of peroxisomes and mitochondria. This pellet was suspended to a concentration of 5 to 10 mg of protein per ml of 1 M SMP (10 to 12 ml total from a 10-liter culture), of which 2.5 ml was loaded over a 32-ml discontinuous sucrose gradient (3.0 ml of 35% sucrose; 6.5 ml each of 40, 43, 46, and 50% sucrose; 3.0 ml of 60% sucrose [all wt/wt]) (26). These gradients were centrifuged at 4°C for 6 h at 27,000 rpm in a Beckman SW28 rotor (100,000 × *g* [average]). The gradient was fractionated by hand from the top. Samples were frozen in liquid nitrogen and stored at −70°C for enzyme analysis or fixed with glutaraldehyde for electron microscopic examination, or the peroxisomal fractions were immediately processed for peroxisomal membrane purification (see below).

Electron microscopy. Immunocytochemical labeling was performed on ultrathin sections of intact cells by using antibodies against malate synthase from *C. tropicalis* (from M. Ueda, Kyoto, Japan) and detected via protein A-gold conjugates (17). The cells were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 90 min at 0°C, dehydrated in a graded series of ethanol, and embedded in Lowicryl K4M. Subcellular fractions were fixed in 5% (vol/vol) glutaraldehyde–0.1 M sodium cacodylate buffer (pH 7.2) for 90 min at 0°C and postfixed in a mixture of 0.5% OsO_4 and 2.5% $K_2Cr_2O_7$ in the same buffer for 60 min at 0°C. The samples were dehydrated in a graded series of ethanol and embedded in Epon 812. Ultrathin sections were cut with a diamond knife and examined in a Phillips EM 300 electron microscope.

Purification of peroxisomal membrane proteins. The peroxisomal fractions from the sucrose gradient described above were diluted fourfold by the addition of TI buffer (10 mM Tris chloride [pH 8], 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine chloride, 5 μg of both leupeptin and aprotinin per ml; all chemicals were from Sigma Chemical Co.). The suspension was centrifuged for 90 min at 50,000 rpm in a Beckman Ti70 rotor. The pellet was suspended in a minimal volume of cold TI buffer (typically, 200 μl), and sodium carbonate was added to a final concentration of 0.1 M carbonate. This mixture was incubated on ice for 1 h with occasional mixing. Membranes were then recovered by centrifugation for 2 h at 50,000 rpm in a Ti 70 rotor. The peroxisomal membranes were further purified from the pellet, which was suspended in 300 μl of TI buffer and applied to a 3.8-ml continuous 20 to 40% (wt/vol) sucrose gradient in TI buffer with a 200-μl cushion of 50% sucrose. The gradients were centrifuged in a Beckman SW60 rotor at 2°C for 18 h at 50,000 rpm; 300-μl fractions were then collected.

Protein gels and immunoassays. Nine percent polyacrylamide gels (35) were used for protein separation. For analysis of the gradient fractions and also of preliminary steps, the proteins were precipitated in 10% trichloroacetic acid. Protein precipitates were washed twice in cold acetone and dissolved in loading buffer (35). Protein gels were fixed and stained as previously described (25). Immunoblotting was performed by standard methods (61), detected by using goat anti-mouse or anti-rabbit second antibodies conjugated to horseradish peroxidase (Organon Teknika), and visualized with 4-chloro-1-naphthol. For affinity purification of antibodies, proteins that were electrophoretically transferred to nitrocellulose were visualized by staining the nitrocellulose in a solution of 0.2% Ponceu S dye, 3% trichloroacetic acid, and 3% sulfosalicylic acid. The protein bands were excised into small strips which were destained in PBS (150 mM NaCl, 20 mM HPO_4 [pH 7.5]). These strips were preincubated in 5% (wt/vol) bovine serum albumin (fraction V)–10 mM Tris chloride (pH 7.4)–150 mM NaCl for 1 h minimum before being washed three times in PBS. The strips were incubated with rabbit antiserum to peroxisomal membrane proteins from *C. boidinii* (25). After similar washes in PBS, specifically bound antibodies were eluted from the nitrocellulose strips with 0.2 M glycine–1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (pH 2.7), which was subsequently neutralized by addition of an equal volume of 100 mM Tris chloride (pH 8.0).

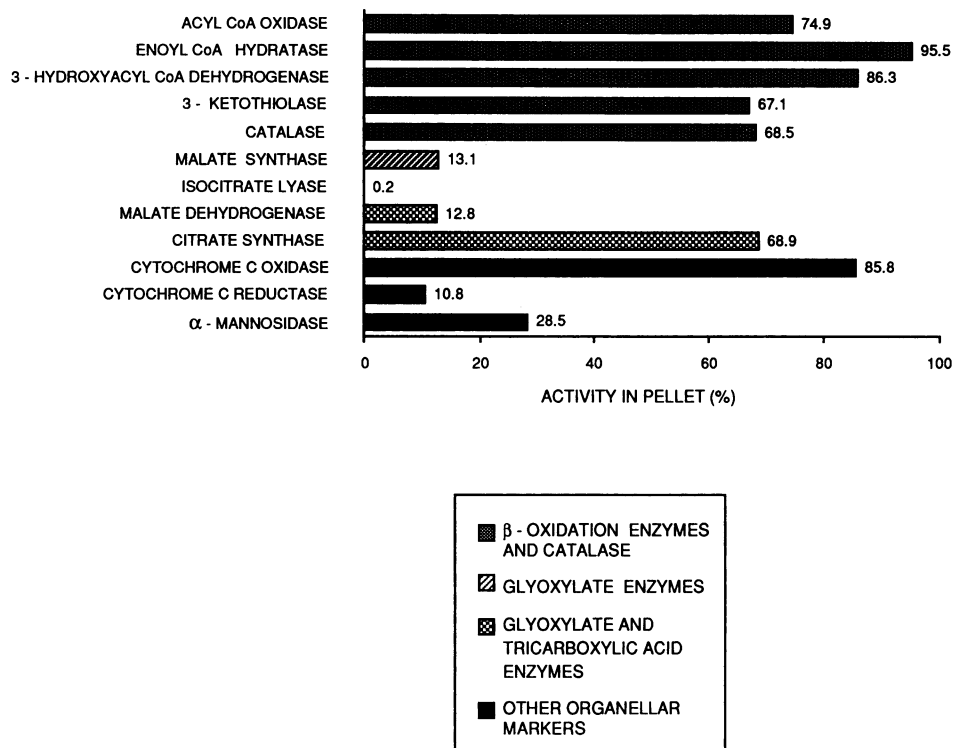**Enzyme assays.** Enzyme activities are expressed in micro-

ACYL CoA OXIDASE — 74.9
ENOYL CoA HYDRATASE — 95.5
3 - HYDROXYACYL CoA DEHYDROGENASE — 86.3
3 - KETOTHIOLASE — 67.1
CATALASE — 68.5
MALATE SYNTHASE — 13.1
ISOCITRATE LYASE — 0.2
MALATE DEHYDROGENASE — 12.8
CITRATE SYNTHASE — 68.9
CYTOCHROME C OXIDASE — 85.8
CYTOCHROME C REDUCTASE — 10.8
α - MANNOSIDASE — 28.5

0   20   40   60   80   100

ACTIVITY IN PELLET (%)

■ β - OXIDATION ENZYMES AND CATALASE

☑ GLYOXYLATE ENZYMES

▩ GLYOXYLATE AND TRICARBOXYLIC ACID ENZYMES

■ OTHER ORGANELLAR MARKERS

FIG. 1. Distribution of enzymes between the crude peroxisomal-mitochondrial pellet ($22,000 \times g$ [maximum] for 20 min) and the supernatant. For details on the preparation, see Materials and Methods. Specific activities for these enzymes are summarized in Table 1. Enzymes are coded for metabolic pathways as shown.

moles per minute for all enzymes except cytochrome c oxidase, cytochrome c reductase, α-mannosidase, acyl-CoA oxidase, 3-ketothiolase, and malate synthase, whose activities are expressed in nanomoles per min. Specific activities are expressed as units of activity per milligram of protein (6). Published methods were used for measurement of cytochrome c oxidase (59), α-mannosidase (69), NADPH cytochrome c reductase (51), citrate synthase (CS) (56), isocitrate lyase (13), catalase (38), 3-ketothiolase with acetoacetyl-CoA as the substrate (41), 3-hydroxyacyl-CoA dehydrogenase with acetoacetyl-CoA as the substrate (63), enoyl-CoA hydratase with crotonyl-CoA as the substrate (57), and acyl-CoA oxidase with decanoyl-CoA as the substrate (16). Measurements of total malate dehydrogenase (MDH) activity (40) and inhibition of the isozyme MDH-2 by 5 mM cetyl (hexadecyl)trimethylammonium bromide (CTAB) were done by a slight modification of the published procedure (55).

For measurement of malate synthase, glyoxylate-dependent release of CoA was measured in a 1-ml volume. Reaction conditions were 100 mM Tris chloride (pH 8)–5 mM MgCl$_2$–100 μM acetyl-CoA–5 mM sodium glyoxylate. Reactions were initiated by addition of enzyme samples and, after incubation at timed intervals, were stopped by addition of trichloroacetic acid to a 5% final concentration. The samples were spun in a microcentrifuge, and 1 ml of the supernatant was neutralized with 200 μl of 1 M sodium carbonate. A 125-μl volume of 1 mM dithionitrobenzoate was added, and the anion released from the thiol reagent was quantitated at 412 nm (ε, 13,600 M^{-1} cm^{-1}).

RESULTS

Isolation of peroxisomes from *S. cerevisiae*. As a first step in the analysis of the composition of peroxisomes from oleate-grown cells of *S. cerevisiae*, the distribution of various enzyme activities in a crude peroxisomal-mitochondrial pellet ($22,000 \times g$ [maximum]) and the corresponding supernatant was analyzed (Fig. 1). In addition to 86% of a mitochondrial marker, cytochrome c oxidase, 68% of catalase activity and 65 to 96% of the enzymes of the beta-oxidation pathway were located in the pellet fraction. Vacuolar and microsomal membranes were found principally in the supernatant as determined by the marker enzymes α-mannosidase and NADPH cytochrome c reductase, respectively. The two enzymes unique for the glyoxylate pathway, malate synthase and isocitrate lyase, were also found primarily in the supernatant, although 10 to 15% of malate synthase was consistently associated with the pellet. Analysis of MDH and CS was complicated by the presence of two isozymes for these activities, each encoded by separate genes. One set of these isozymes (MDH-1 and CS-1) is located in the mitochondrion, where they function in the tricarboxylic acid cycle, while the other set (MDH-2 and CS-2) is extramitochondrial. The distribution of total CS activity was consistent with published data (31), with 69% partitioning in the pellet. However 90% of the total MDH activity was found in the supernatant in our experiments. This was primarily MDH-2 activity and not leakage of MDH-1 activity from mitochondria, since 95% of this soluble activity was inhibited by the detergent CTAB, a specific inhibitor of MDH-2 (55). There was also CTAB-inhibitable activity in the pellet, although quantitation was difficult because of activation of MDH-1 by the surfactant (see below).

The $22,000 \times g$ pellet was layered onto a 35 to 60% (wt/wt) discontinuous sucrose gradient, which was centrifuged to separate the organelles (see Materials and Methods for further details). The peroxisomal fractions were defined by
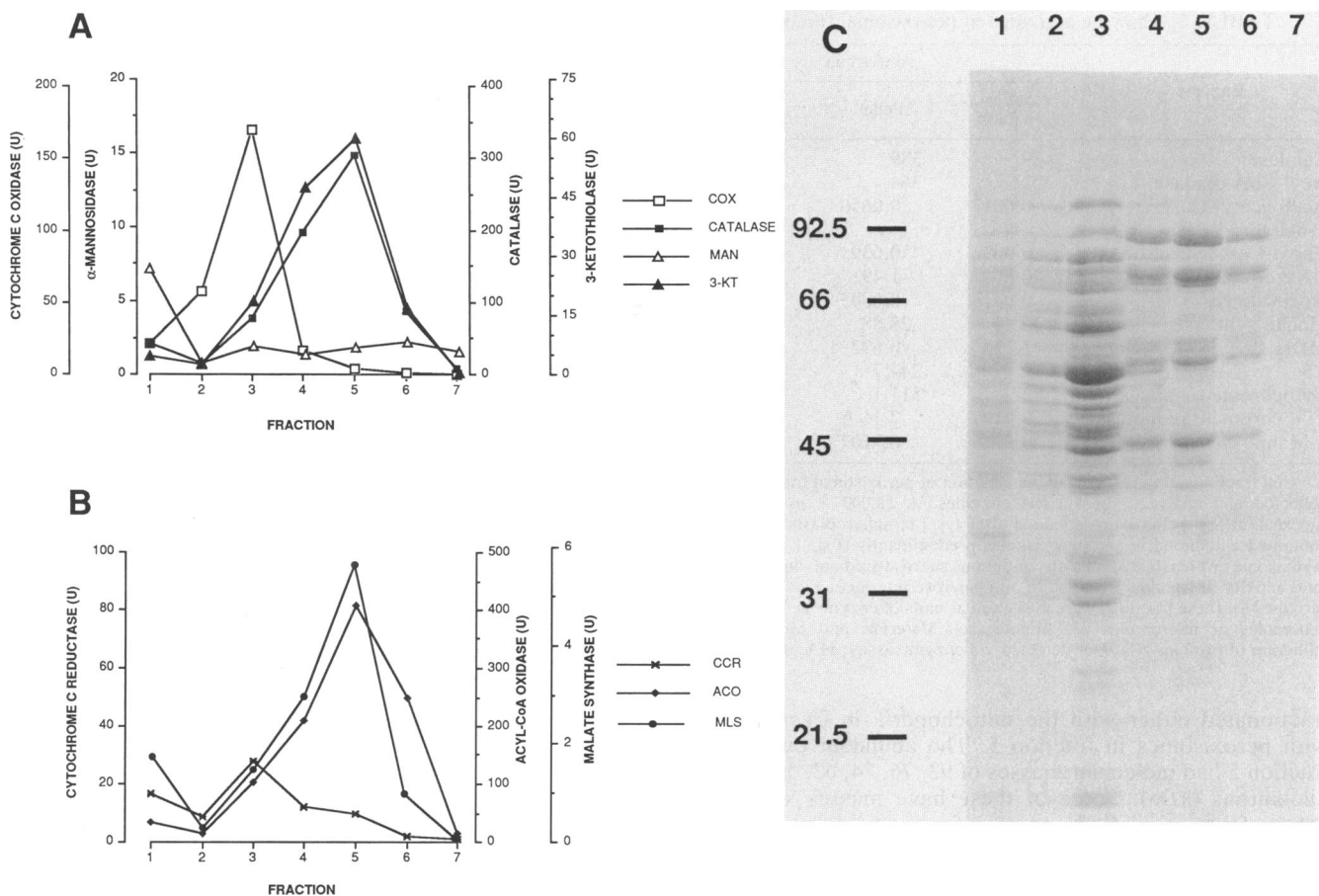
FIG. 2. Peroxisomal fractionation on a discontinuous sucrose gradient. Data are from the experiment whose results are reported in Fig. 1 and Table 1. The fractions represent interfaces between sucrose concentrations as follows: 1, 0 to 35% sucrose; 2, 35 to 40% sucrose; 3, 40 to 43% sucrose; 4, 43 to 46% sucrose; 5, 46 to 50% sucrose; 6, 50 to 60% sucrose; 7, 60% sucrose and pellet. For details, see Materials and Methods. (A and B) Distribution of enzyme activities from the gradient. All enzyme activities are expressed in units. The activity in the gradient was normalized to 2 mg of gradient protein. (C) Protein profile of gradient fractions. A portion (0.5%) of each fraction was loaded such that approximately 10 μg of fraction 5 (peroxisomal peak) could be visualized. The gel was fixed and stained in 0.25% Coomassie brilliant blue–30% methanol–10% acetic acid. The numbers to the left indicate molecular sizes in kilodaltons. Abbreviations: MAN, α-mannosidase; 3-KT, 3-ketothiolase; COX, cytochrome *c* oxidase; ACO, acyl-CoA oxidase; CCR, NADPH cytochrome *c* reductase; MLS, malate synthase.

the colocalization of catalase and the beta-oxidation enzymes acyl-CoA oxidase and 3-ketothiolase (Fig. 2A and B). The beta-oxidation enzymes and catalase were enriched 4- to 17-fold over the crude pellet (Table 1). The peroxisomes banded primarily at the interface between 46 and 50% sucrose (fraction 5) and represented 15 to 20% of the gradient protein by mass. Pelletable malate synthase colocalized with the peroxisomal fractions (Fig. 2) and had a concomitant increase in specific activity over the crude pellet (Table 1). Immunocytochemical experiments on thin sections with polyclonal antibodies to malate synthase from *C. tropicalis* confirmed a peroxisomal location for malate synthase in *S. cerevisiae* grown on oleate (Fig. 3). Residual isocitrate lyase activity from the pellet was observed in the top gradient fraction only (data not shown); there was no detectable activity in the peroxisomal fractions.

Mitochondria were the major organellar component on the gradients, banding between the 35 and 40% sucrose layers (fraction 3), and represented approximately 60% of the gradient protein mass. Despite the preponderance of mitochondria on the gradient, less than 2% of that organelle was

present in the peroxisomal fractions, as judged by the marker enzyme cytochrome *c* oxidase. As observed by light microscopy, unbroken spheroplasts did not appear to enter the gradient and were found in fraction 1. Similarly, vacuolar membranes, as measured by α-mannosidase, were observed at the top of the gradient (Fig. 2). Low NADPH cytochrome *c* reductase activity was observed throughout the gradient. The apparent increase in specific activity for this enzyme in the peroxisomal peak over the crude pellet (Table 1) was due to a 10-fold increase in total activity recovered from the sucrose gradient compared with the amount applied (all other enzymes similarly assessed were within 75 to 150%); the activity in the peroxisomal fraction was among the lowest on the gradient (the range was 50 to 300 U/mg), suggesting that there was minimal presence of microsomes with the peroxisomal fractions. This was confirmed by electron microscopic analysis of peroxisomal fractions, which revealed little microsomal contamination (see Fig. 6A and B).

Two distinct profiles of proteins were observed on gels of these fractions (Fig. 2C), indicating that most proteins co-

TABLE 1. Enzyme activities in peroxisomal fractions[a]

Enzyme	Activity in:		
	Super-natant	Pellet	Peroxi-somal peak
Catalase	39	389	2,129
Acyl-CoA oxidase	22	364	2,843
Acetoacetyl-CoA dehydrogenase	0.0017	0.0650	0.2569
Enoyl-CoA hydratase	0.003	0.639	9.108
3-Ketothiolase	0.86	1.49	25.10
Isocitrate lyase	0.0438	0.0005	ND
Malate synthase	32.10	28.88	48.14
MDH	1.118	0.692	NA
CS	19.4	244.7	NA
Cytochrome c oxidase	12.7	511.1	30.1
Cytochrome c reductase	9.91	7.16	68.34
α-Mannosidase	0.2976	0.7107	0.7630

[a] Cell fractions obtained during the isolation of peroxisomal fractions were collected and analyzed for various enzymes. A 22,000 × g (maximum) supernatant and pellet were obtained after lysis of spheroplasts. The pellet contained mitochondria and peroxisomes predominantly (Fig. 1 and 2). The peroxisomes were isolated on a discontinuous sucrose gradient, with a peak in total activity at fraction 5 (the 46 to 50% sucrose interface; Fig. 2). Specific activities for these fractions were measured as units of enzyme activity (either nanomoles or micromoles per minute; see Materials and Methods) per milligram of protein. ND, Not detected via enzyme assay; NA, not assayed.

fractionated either with the mitochondria in fraction 3 or with peroxisomes in fraction 5. The abundant proteins in fraction 5 had molecular masses of 92, 76, 74, 62, 59, and 44 kilodaltons (kDa). Some of these have masses similar to those of beta-oxidation enzymes from other organisms (2, 43–45). Malate synthase has been purified from *S. cerevisiae* and has a subunit molecular mass of 63 kDa (14), while catalase A has a predicted molecular mass of 59 kDa on the basis of the gene sequence (9). However, no further attempt was made to identify specific enzymes with protein bands.

Induction of peroxisomes. To investigate whether peroxisomes could be clearly detected biochemically in cultures in which the organelle was not induced, cells were grown on glycerol, a non-catabolite-repressing carbon source which does not induce peroxisome proliferation (confirmed by electron microscopy; data not shown). In addition, cells
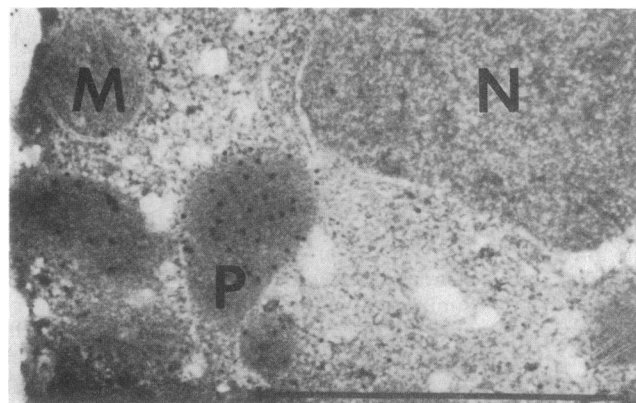

FIG. 3. Detection of malate synthase in peroxisomes by immunoelectron microscopy. Binding of antibodies to the enzymes from *C. tropicalis* (obtained from M. Ueda, Kyoto University, Kyoto, Japan) was detected via protein A-gold conjugates. For details, see Materials and Methods. Bar, 1 μm. M, Mitochondrion; N, nucleus; P, peroxisome.

were grown in a medium combining glycerol and oleate to observe peroxisome proliferation when both substrates could be metabolized. In this latter case, the growth rate of the culture was similar to the growth rate on glycerol alone but substantial oleate uptake and metabolism had also occurred, as determined by substantial lipid release after lysis of spheroplasts and by induction of beta-oxidation and glyoxylate enzymes (see below).

Organelles from these growth conditions were fractionated and compared with those from a culture grown on oleate alone. After normalization of enzyme activities for total protein loaded onto the sucrose gradients, several aspects of peroxisome proliferation were apparent. Peroxisomes from cultures grown on glycerol were detected by coinciding peaks of catalase, acyl-CoA oxidase, and malate synthase activities (Fig. 4A). These enzyme levels were much lower than when growth occurred on oleate. In addition, the peroxisomal peak was found at a lower sucrose concentration than the corresponding peaks from oleate-grown cultures (between 40 and 46% sucrose [fractions 3 and 4] instead of between 40 and 50% sucrose [fractions 4 and 5]; Fig. 4A and C). The peroxisomal peak from glycerol-grown cells was closer to the mitochondrial peak. Separation of the peroxisomal and mitochondrial organelles was not significantly improved by longer centrifugation times, indicating that these peroxisomes were at their buoyant density (data not shown). Peroxisomal fractions from a culture grown on glycerol plus oleate had enzyme levels intermediate between those of parallel samples from cultures grown on the individual carbon sources but with a buoyant density similar to that of the organelle from the oleate-grown culture (Fig. 4B).

Localization of MDH-2. The localization of MDH with peroxisomes was also investigated. The activity is expressed from two distinct genes, which express the mitochondrial isozyme (MDH-1) and the nonmitochondrial or cytosolic isozyme (MDH-2; 8, 40). Analysis of total MDH on gradients separating mitochondria from peroxisomes is shown in Fig. 4C. In this experiment, 80% of the total MDH was observed in the soluble fraction and most of the remaining pelletable activity was observed with the peroxisomal fractions. Several experiments were performed to determine whether the MDH-2 isozyme was associated with the peroxisomal fractions.

Mitochondrial MDH activity had a pH-activity profile different from that of MDH in either the soluble or peroxisomal fraction. The ratio of mitochondrial MDH activity at pH 7.5 to 6.4 was approximately 9, while the ratios of activities for MDH in both the supernatant and peroxisomal fractions were approximately 2. When peroxisomes from a mutant lacking MDH-2 (*mdh2*) were analyzed, the MDH activity appeared solely in the mitochondrial fractions and was not significantly associated with the peroxisomes (data not shown). Another strain containing an *mdh1* deletion did not grow on oleate. Fractionation of the cultures was poor, and abundant and heavy peroxisomes were not isolated.

MDH isozymes were also distinguished by using the surfactant CTAB (55). This compound inhibits purified yeast MDH-2 while slightly stimulating MDH-1 (J. S. Steffan and L. McAlister-Henn, personal communication). We analyzed CTAB-inhibitable MDH activity in several cellular fractions. The MDH in the 22,000 × g supernatant was almost entirely MDH-2, since the activity was 98% inhibited by CTAB (Table 2). Similarly, 60% of the activity in the mitochondrial-peroxisomal pellet was inhibited, indicating a large amount of MDH-2 in this fraction. When CTAB inhibition was analyzed on sucrose gradient fractions, the most sensitive
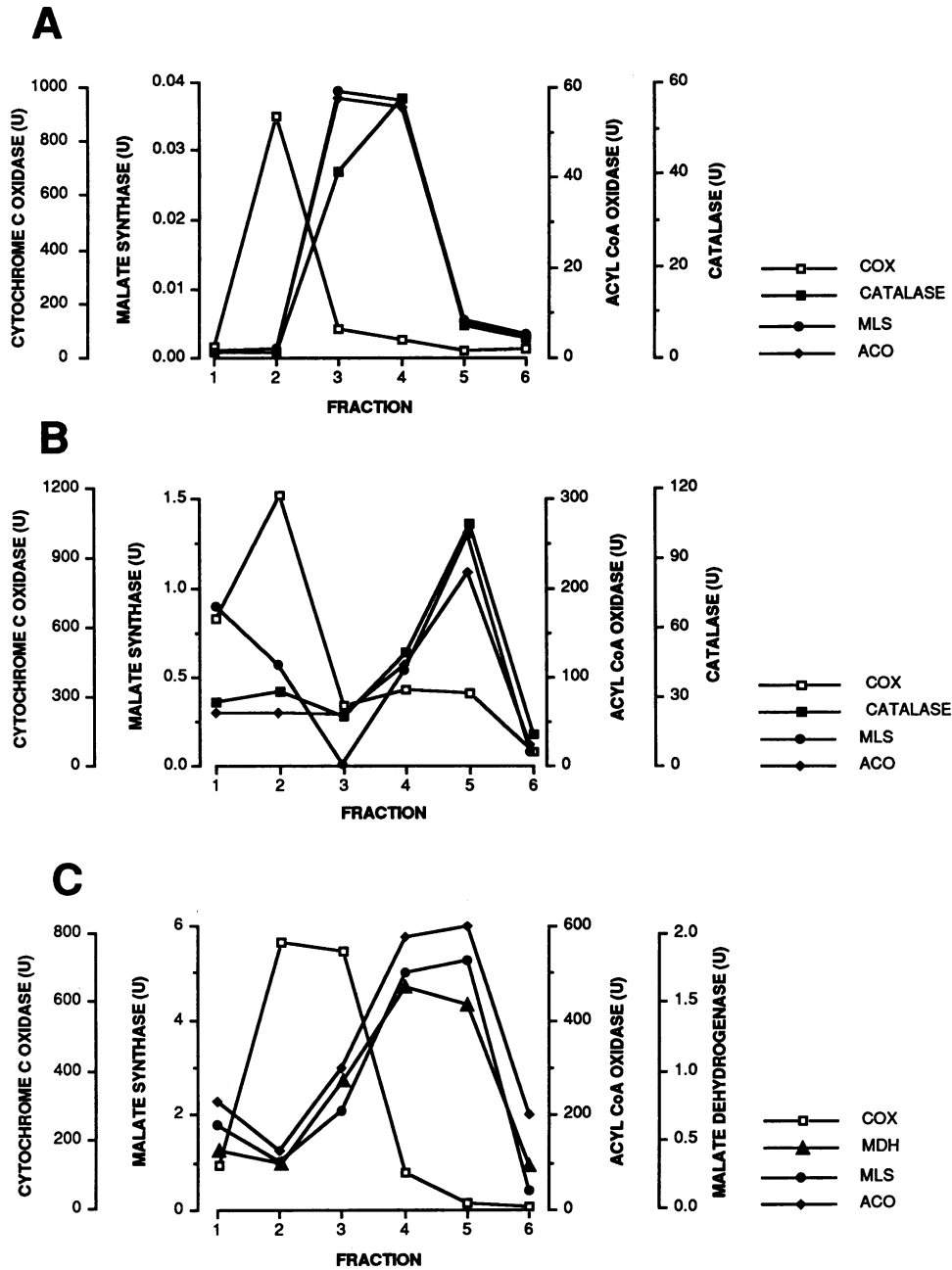
FIG. 4. Distribution of enzyme activities on discontinuous sucrose gradients from cultures grown on different carbon sources. Gradients were fractionated and analyzed for enzyme activities and protein as described in Materials and Methods. Activities from each experiment were normalized for 2 mg of total gradient protein. Panels: A, culture grown on YPG; B, culture grown on YPG plus 0.1% oleate; C, culture grown on semisynthetic 0.1% oleate. MDH, Malate dehydrogenase; other abbreviations are the same as in Fig. 2.

fractions were those containing peroxisomes (fractions 4 and 5). Activity in fraction 2, the mitochondrial peak, was stimulated by CTAB, indicating the presence of a large amount of MDH-1. Intermediate fractions probably reflected combined effects of MDH-1 stimulation and MDH-2 inhibition.

The relative proportion of these isozymes estimated by CTAB sensitivity in these fractions was confirmed by using antisera specific for each isozyme. Antiserum to MDH-1 detected the isozyme primarily in the mitochondrial frac-

tions (Fig. 5), while antiserum specific for MDH-2 detected that isozyme in the peroxisomal and cytosolic fractions. These results indicate that MDH-2 can be found localized with peroxisomes. However, like malate synthase, MDH-2 appears to leak readily from the organelle during isolation. The pattern of MDH-2 distribution between the cytosolic and peroxisomal locations was consistent in every experiment reported here and was also consistent with the distribution of malate synthase.

Membrane proteins of peroxisomes. Purified peroxisomes

TABLE 2. Inhibition of MDH activity by CTAB[a]

Fraction	% Activity in 5 mM CTAB
22,000 × g fractions	
Supernatant	2.1
Pellet	39.2
Gradient fractions	
1	66.3
2	223.4
3	95.0
4	46.2
5	36.4
6	59.7

[a] The effects of CTAB on MDH activities in cell fractions consisting of a 22,000 × g (maximum) supernatant and pellet and sucrose gradient fractions derived from the pellet (shown in Fig. 4C) were analyzed. MDH (0.04 U of activity) was treated with 5 mM CTAB in 0.15 ml. MDH activity was measured after 2.5 and 5 min of incubation at room temperature with the surfactant. The average of these rates was compared with a parallel rate obtained from control samples incubated without CTAB. Inhibition (or stimulation) of MDH activity by CTAB was measured as a percentage of the control activity for each fraction.

similar to those described in Fig. 1 were used to identify the peroxisomal membrane proteins of *S. cerevisiae*. For this analysis, two fractions were studied to rule out mitochondrial contamination. Fraction 5 contained the peak of peroxisomal markers and approximately 1.5% of the gradient cytochrome c oxidase activity (i.e., mitochondria). Fraction 6 was also examined because it was more highly enriched for peroxisomes; the ratio of mitochondrial to peroxisomal activities was 10-fold less than in fraction 5. The relative purity of these fractions was confirmed by electron microscopy (Fig. 6A and B). The organelles appeared somewhat hollow. Electron-dense material characteristic of peroxisomes (1, 24) was seen adhering to the inner surface of most peroxisomes. Vesicular structures of unknown composition were also seen inside the peroxisomes.

Fractions 5 and 6 were extracted with sodium carbonate, and the carbonate-insoluble material was purified by equilibrium centrifugation on continuous sucrose gradients.

FIG. 5. Detection of MDH-2 in peroxisomes. Portions of the pellet and supernatant fractions (0.1%) and gradient fractions (0.5%) analyzed in Fig. 4C and Table 2 were probed with antisera specific for MDH isozymes as indicated (obtained from L. McAlister-Henn, University of California, Irvine). S, Supernatant; P, pellet.

There was a slight but reproducible difference between the apparent buoyant densities of the membranes from fractions 5 and 6 (fraction 6 was slightly heavier). The same proteins of 32, 31, and 24 kDa cofractionated in both samples (Fig. 6C and D), indicating that they exist in the same membrane. This relatively simple protein composition appears to be characteristic of peroxisomal membranes. On comparison to an identical fraction from *C. boidinii* (25), this similarity was readily apparent (Fig. 7). The abundant *Candida* proteins (PMP47, PMP32, and PMP31) were observed from peroxisomal membranes of *C. boidinii* grown on methanol, oleate, and D-alanine (Goodman et al., in press). *C. boidinii* peroxisomes also contain a membrane-associated protein, PMP20, which is specific to methanol metabolism (23). The 31- and 32-kDa proteins from *S. cerevisiae* nearly comigrated with PMP31 and PMP32 from *C. boidinii*, while the 24-kDa protein of *S. cerevisiae* appeared to be distinct. The less abundant proteins observed in these fractions in the 60- to 80-kDa range were characteristic of peroxisomal membrane extracts from oleate-grown cultures from both yeasts (Goodman et al., in press) and likely represent incomplete extraction of beta-oxidation proteins by carbonate (62). Recently the peroxisomal membrane proteins from *C. tropicalis* were reported (42). Proteins of 34, 29, and 24 kDa were observed in carbonate-insoluble extracts of those peroxisomes. These proteins are almost identical in apparent electrophoretic mobility to the proteins in gradient-purified membranes of *S. cerevisiae*.

Because of the similarities in the sizes and extraction properties of these membrane proteins, it was logical to envision that the proteins also performed similar functions in the peroxisomes and would likewise be similar in sequence and antigenic properties. Unfortunately, no functions for these proteins have been demonstrated. Attempts to demonstrate antigenic similarities between these proteins have been unsuccessful. Monoclonal or affinity-purified polyclonal antibodies to PMP47 of *C. boidinii* (25) do not recognize proteins in *S. cerevisiae* peroxisomes. Similarly, affinity-purified antibodies to PMP32 or PMP31 do not recognize proteins in *S. cerevisiae* (data not shown). Interestingly, affinity-purified antiserum to *Candida* PMP31 does not recognize PMP32, and anti-PMP32 only poorly recognizes PMP31, even though the two *Candida* proteins are similar in primary structure (J. M. Goodman, R. A. Lark, and C. Slaughter, unpublished data). Thus, these antibodies are probably too specific to cross-react with the putative homologs of *S. cerevisiae*. Antibodies which recognize both PMP31 and PMP32 will be useful to probe the similarities between proteins of the two yeasts.

DISCUSSION

The attractiveness of *S. cerevisiae* as a genetic system to study many aspects of cell biology are well-known and prompted our interest in using this yeast to study peroxisomal biogenesis, structure, and metabolism. In this study, we analyzed the composition of peroxisomes from this organism in detail. This analysis was made feasible by the proliferation of peroxisomes after growth on oleic acid as a carbon source (68) and by adaptation of fractionation methods developed for *C. boidinii*. Our results provide evidence that at least some of the enzymes of the glyoxylate pathway, which are induced by oleic acid and are necessary for growth on that substrate, are localized in peroxisomes with the enzymes of beta-oxidation. These results are based on cell fractionation and electron microscopy.
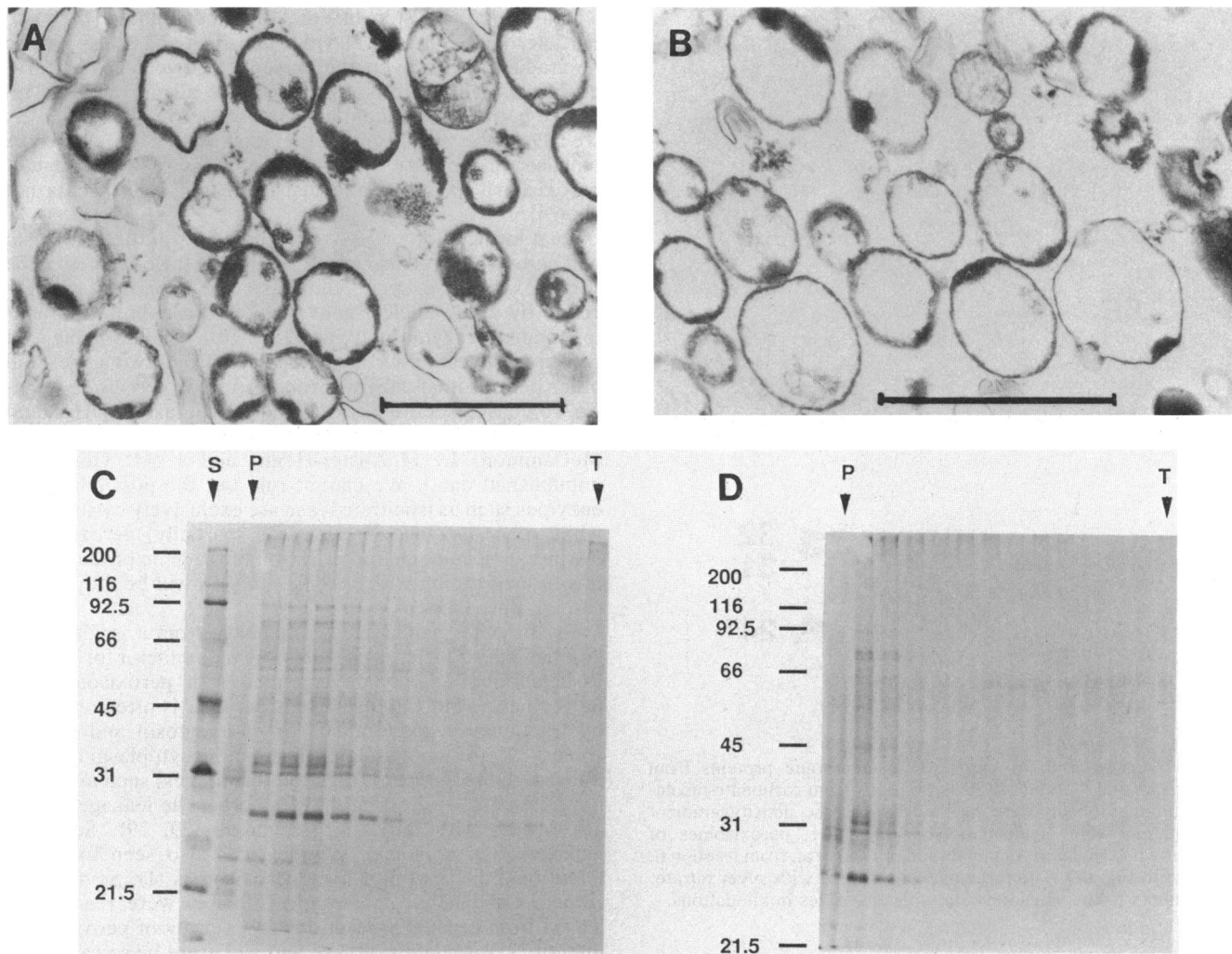
FIG. 6. Purification of peroxisomal membrane proteins. (A and B) Electron micrographs of peroxisomal peak fractions 5 (A) and 6 (B) from an experiment similar to that shown in Fig. 1 and 2. Bar, 1 μm. (C and D) Purified peroxisomal membrane proteins after extraction with sodium carbonate. Carbonate-insoluble proteins were centrifuged for 18 h at 50,000 rpm in a Beckman SW60 rotor through a 20 to 40% sucrose gradient (see Materials and Methods for further details). The gradients were fractionated and precipitated with tricholoroacetic acid. Protein profiles of these gradients were stained with silver nitrate. Panels: C, fraction 5; D, fraction 6. P, Gradient pellet; T, top of gradient; S, protein standards. The numbers to the left indicate molecular sizes in kilodaltons.

Analysis of cell fractions revealed two classes of enzymes based on the avidity with which they were associated with the peroxisomes. Enzymes in the first class were efficiently pelleted with crude peroxisomal membranes, in the range of 65 to 95% of the total activity (Table 1). This includes enzymes of the beta-oxidation pathway and catalase. In *S. cerevisiae*, there are two genes that encode catalases, *CTT1* and *CTA1* (53). Expression of catalase A, encoded by *CTA1*, is induced by growth on oleate; by use of a *ctt1* mutant, catalase A was demonstrated to be localized in peroxisomes (54). Catalase T is soluble (54) and presumably represents a significant portion of the enzyme activity in the supernatant.

The glyoxylate pathway enzymes were in a second class of peroxisomal enzymes which were inefficiently associated with peroxisomal pellets, in the range of 0 to 20% (Table 1). We were not able to observe isocitrate lyase with the organelle biochemically; immunoelectron microscopy with antiserum to the enzyme showed some reactivity with peroxisomes (M.V., unpublished data), but the definitive exper-

iment may require antibodies with higher affinity. While only 13 to 15% of the total malate synthase was found in the crude peroxisomal-mitochondrial pellet, the enzyme clearly fractionated with peroxisomes on sucrose gradients. This localization was confirmed by immunoelectron microscopy (Fig. 3). Cofractionation of malate synthase with catalase and acyl-CoA oxidase was also observed when organelle proliferation was not induced by oleate (Fig. 4A).

Determination of a peroxisomal localization for MDH-2 was complicated by a mitochondrial isozyme which could be a contaminant in the peroxisomal gradient fractions. When yeast cultures were grown on oleate or acetate, MDH-2 was the major cellular isozyme. Eighty to ninety percent of the total MDH activity was in the cytosolic fraction and was distinguishable as MDH-2 by several criteria, i.e., its sensitivity to the surfactant CTAB (Table 2), loss of cytosolic MDH activity in an *mdh2* mutant, activity at pH 6.5, and use of MDH-2-specific antiserum (Fig. 5). These same parameters were used to identify MDH-2 unambiguously in perox-
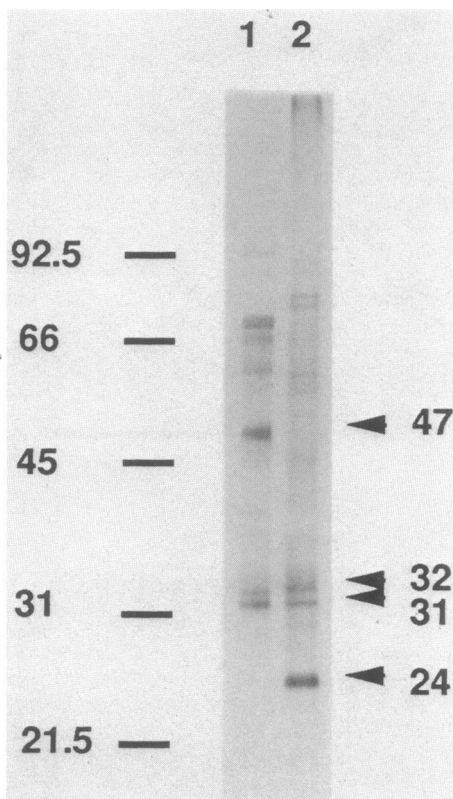
FIG. 7. Comparison of peroxisomal membrane proteins from *S. cerevisiae* and *C. boidinii*. Peak fractions from carbonate-insoluble membranes which were purified by sucrose density gradient centrifugation were electrophoresed. Lanes: 1, peroxisomes of oleate-grown *C. boidinii*; 2, membrane protein peak from fraction 6, as shown in Fig. 6D. The proteins were stained with silver nitrate. The numbers to the sides indicate molecular sizes in kilodaltons.

isomal fractions. Although MDH-2 represented the major portion of total pelletable MDH activity (Fig. 4C), the amount of MDH-2 associated with the peroxisomal fractions was low, on the order of 5 to 10% of total MDH-2.

Similarly, CS activity is encoded by two genes. *CIT1* encodes the mitochondrial isozyme, while *CIT2* encodes the nonmitochondrial CS-2 isozyme (31, 49). A recent report concluded that CS-2 was peroxisomal (37). Analysis of the protein sequence derived from the *CIT2* gene, which encodes CS-2, revealed a carboxy-terminal sequence of SKL, which is a putative peroxisomal targeting signal (27) and is found at the carboxy termini of several peroxisomal proteins. The occurrence of such a signal reinforces the inference of a peroxisomal location for CS-2 on the basis of initial biochemical studies (37).

There are at least three interpretations to our finding that glyoxylate cycle enzymes are poorly associated with peroxisomes when isolated. (i) There may be distinct microbodies for the beta-oxidation enzymes and catalase (i.e., peroxisomes) and for the glyoxylate pathway (i.e., glyoxysomes), and the glyoxysomes are more fragile; (ii) the glyoxylate enzymes may be localized in both the cytoplasm and peroxisomes; or (iii) there may be selective leakage of glyoxylate enzymes from the same organelle.

Filamentous fungi such as *N. crassa* contain two different types of microbodies (32), one containing catalase and the enzymes of the glyoxylate pathway and the other containing

a beta-oxidation system like that of mitochondria. These two particles have different buoyant densities and are separable on gradients. However, the presence of two types of particles in *S. cerevisiae* is unlikely, since all microbodies reported in this species contain catalase (3, 19, 67). Moreover, we have observed the cofractionation of catalase, acyl-CoA oxidase, and malate synthase regardless of the extent of peroxisomal induction and density (Fig. 2 and 4; data not shown).

Dual localization of glyoxylate enzymes in the cytoplasm and peroxisomes is also possible. Two of the enzymes of the tricarboxylic acid cycle, fumarase and aconitase, are encoded by single nuclear genes which also express extramitochondrial enzyme activities (22, 70). While soluble and peroxisomal catalases are expressed from two genes, all other peroxisomal enzymes reported in *S. cerevisiae* (acyl-CoA oxidase, isocitrate lyase, malate synthase, MDH-2, and CS-2) appear to be encoded by single genes (8, 15, 50; M. T. McCammon, L. McAlister-Henn, and J. M. Goodman, unpublished data). We cannot rule out the possibility that enzymes such as isocitrate lyase are exclusively cytoplasmic while other enzymes are (at least partially) peroxisomal. Further molecular characterization of the gene products and in situ localization with specific antisera will be required to resolve this issue.

On the other hand, selective leakage from a single population of microbodies is probably the major factor for the low association of glyoxylate enzymes with peroxisomes reported here. Although catalase in rat liver is often observed by fractionation experiments in both cytosol and peroxisomes (39), the amount determined in the cytoplasm is lower if measured by noncentrifugation techniques, such as digitonin treatment, suggesting that less organelle leakage-breakage occurs with the latter technique (29, 39). Selective leakage of peroxisomal enzymes was also seen following intentional disruption of liver peroxisomes (1). Several enzymes, especially catalase and thiolase, were readily released from organellar fractions in a survey of peroxisomal integrity (1). Malate synthase and isocitrate lyase can differ greatly in their solubilization properties from cotton cotyledon glyoxysomes (7). In *C. boidinii*, we have observed that raising the pH during spheroplast lysis selectively releases the matrix enzymes catalase and dihydroxyacetone synthase before alcohol oxidase; much of the electron-dense ground substance escapes from the peroxisomes at this time (24). The peroxisomes isolated in this study appeared empty compared with the organelles in situ (Fig. 6A and B), although some internal structure was still evident. Since the inside of the organelle may not be homogeneous in composition, it is possible that the glyoxylate enzymes reside in a different peroxisomal location which is more sensitive to leakage during preparation.

The reason(s) for peroxisomal fragility is unknown. It is unlikely that osmotic forces are solely responsible, since mechanical lysis under iso-osmotic conditions did not increase the percentage of glyoxylate enzymes that was pelletable. Membranes of yeast peroxisomes appear smooth in freeze-fracture electron microscopy (67), as if the density of transmembrane proteins were particularly low compared with those of other membrane proteins. Perhaps there are cytosolic factors which stabilize this structure; upon lysis of cells, these factors are diluted. Experiments to test this hypothesis are in progress.

Since it was possible to isolate pure peroxisomes, it was of interest to fractionate the organelle to identify the membrane proteins. Three proteins of 32, 31, and 24 kDa were observed

(Fig. 7). Interestingly, only three peroxisomal membrane proteins from *C. tropicalis*, i.e., 34, 29, and 22 kDa (42), and *C. boidinii*, i.e., 47, 32, and 31 kDa (25; Goodman et al., in press), were also observed. In view of the simplicity of the membrane protein composition and the similarity in apparent molecular masses (especially the proteins of 29 to 34 kDa) among three diverse yeasts, it is not unreasonable to speculate that these proteins are related not only evolutionarily but also functionally.

In a recent analysis of peroxisomes isolated from cultures of *C. boidinii* grown on methanol, oleic acid, or D-alanine, different peroxisomal matrix proteins were induced by the various substrates, while the membrane proteins were the same (Goodman et al., in press). These results indicated that while the peroxisomal matrix proteins function in specific metabolic pathways which are induced by specific substrates, the peroxisomal membrane proteins are more general and function in a substrate-nonspecific manner. Although peroxisomal proteins, from several organisms have been analyzed (10, 21, 25, 28, 42), functions for none of these resident proteins have been specifically defined. The gene that encodes PMP70 from rat liver peroxisomes has been reported (30), and it appears to have some similarity to a diverse family of proteins involved in membrane transport. Since the peroxisomal membrane appears to be permeable to small molecules (34, 66), the necessity for a pore molecule has been proposed. One report suggested that a protein of 22 kDa could serve as a pore (66). When native peroxisomal membranes or Triton-extracted integral membrane proteins reconstituted into liposomes were used, the diameter of the peroxisomal channel was recently calculated to be 1.7 nm, which is the same magnitude as that of mitochondrial porin (36). It is also of interest that several integral membrane proteins similar in size (30 to 35 kDa) to the peroxisomal membrane proteins observed in several yeasts have been identified in mitochondria. These are the various solute carrier proteins of the inner membrane (ATP-ADP carrier, phosphate carrier, 2-oxoglutarate carrier, and dicarboxylic acid carrier) and porin in the outer membrane (33). Whether any of the three membrane proteins identified in *S. cerevisiae* peroxisomes functions in membrane transport in ways similar to those in the mitochondrial membranes is a subject currently under investigation.

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