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Citrate Synthase Encoded by the *CIT2* Gene of *Saccharomyces cerevisiae* Is Peroxisomal

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The product of the *CIT2* gene has the tripeptide SKL at its carboxyl terminus. This amino acid sequence has been shown to act as a peroxisomal targeting signal in mammalian cells. We examined the subcellular site of this extramitochondrial citrate synthase. Cells of *Saccharomyces cerevisiae* were grown on oleate medium to induce peroxisome proliferation. A fraction containing membrane-enclosed vesicles and organelles was analyzed by sedimentation on density gradients. In wild-type cells, the major peak of citrate synthase activity was recovered in the mitochondrial fraction, but a second peak of activity cosedimented with peroxisomes. The peroxisomal activity, but not the mitochondrial activity, was inhibited by incubation at pH 8.1, a characteristic of the extramitochondrial citrate synthase encoded by the *CIT2* gene. In a strain in which the *CIT1* gene encoding mitochondrial citrate synthase had been disrupted, the major peak of citrate synthase activity was peroxisomal, and all of the activity was sensitive to incubation at pH 8.1. Yeast cells bearing a *cit2* disruption were unable to mobilize stored lipids and did not form stable peroxisomes in oleate. We conclude that citrate synthase encoded by *CIT2* is peroxisomal and participates in the glyoxylate cycle.

Saccharomyces cerevisiae contains two forms of citrate synthase, which are encoded by distinct nuclear genes (14, 20, 21, 25). DNA sequence analysis reveals that the two isozymes are similar in size and primary structure except at their amino termini. The gene for mitochondrial citrate synthase (*CIT1*) encodes a typical targeting peptide for mitochondrial transport. The *CIT2* product lacks this amino-terminal leader and contains serine-lysine-leucine (SKL) as a carboxy-terminal tripeptide. Despite their similarity in amino acid sequence, antibodies raised against mitochondrial citrate synthase do not recognize the extramitochondrial enzyme. Extramitochondrial citrate synthase can also be distinguished by its sensitivity to alkaline pH (21).

Insertion mutations have been generated to inactivate the *CIT1* and *CIT2* genes (14, 20). Cells lacking mitochondrial citrate synthase grow normally in glucose-containing media (15). On nonfermentable carbon sources, *cit1* cells exhibit a prolonged lag phase, and they do not grow in media with acetate as the carbon source. Cells in which the extramitochondrial isozyme is inactive have no obvious phenotype, whereas cells lacking both isozymes are glutamate auxotrophs and grow very slowly on nonfermentable carbon sources.

In some organisms, extramitochondrial citrate synthase is known to participate in the glyoxylate cycle. This metabolic pathway permits many plants and fungi to make a net conversion of fatty acids to carbohydrates. The glyoxylate cycle enables yeasts to utilize ethanol and other two-carbon compounds as a sole carbon source (9). In plants, the enzymes of the glyoxylate pathway are sequestered in a specialized peroxisome called the glyoxysome. Such microbodies contain a unique set of fatty acid oxidation enzymes found commonly in peroxisomes as well as the glyoxylate cycle enzymes that channel the resulting acetyl coenzyme A (acetyl-CoA) into carbohydrate synthesis.

Recently, the induction of peroxisomes was demonstrated in *S. cerevisiae* (6, 28). By growing yeast cells on oleate medium, we have induced peroxisomes and determined that extramitochondrial citrate synthase copurifies with peroxisomes, suggesting that it is involved in the glyoxylate pathway.

MATERIALS AND METHODS

Yeast strains. The yeast strains used were galactose-fermenting derivatives of strains GRF18 (*MATα his3-11,3-15 leu2-3, 2-112 can*^{r}), GK1 (*MATα his3-11,3-15 leu2-32-112 can*^{r} *CIT1*::*LEU2*), and BWG1-7a-U (*MATα ade1-100 his4-519 leu2-3,2-112 ura3-52 CIT2*::*URA3*).

Culture conditions and cell fractionation. Peroxisomes were induced by growing yeast cells for 24 h in a medium consisting of 0.3% yeast extract, 0.5% peptone, 0.5% potassium phosphate (pH 6), 0.05% galactose, 0.1% oleic acid, and 0.2% Tween-80 at 30°C with vigorous agitation.

Cells were converted to spheroplasts by using Zymolyase and were disrupted in 0.6 M sorbitol–5 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-KOH (pH 6.0)–0.5 mM EDTA, using a Dounce homogenizer. After removal of nuclei and unbroken cells by centrifugation at 1,500 × *g*, an organelle fraction was prepared by centrifugation at 27,000 × *g*. The pellet was suspended in 0.24 M sucrose–5 mM MES-KOH (pH 6.0)–1 mM EDTA, and a portion was layered on a gradient consisting of layers of 17% (wt/vol), 25%, and 35% Nycodenz [5-(*N*-2,3-dihydroxypropylacetamido)-2,4,6-triiodo-*N'N'*-bis(2,3-dihydroxypropyl)-isothalamide] made in the same buffer. Centrifugation was carried out in a Beckman SW41 rotor at 28,000 rpm for 2 h. The distribution of enzymes between subcellular fractions was calculated according to de Duve (5). Details of the method will be published elsewhere (V. Hines, manuscript in preparation).

Enzyme assays and antibody blots. Acyl-CoA oxidase (22), catalase (2), citrate synthase (18), cytochrome *c* oxidase (30), malate dehydrogenase (29), and fumarase (19) activities were measured by published procedures. Protein concentra-

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tions were estimated by the method of Bradford (3). Rabbit antibodies were prepared against purified yeast mitochondrial citrate synthase as described by Hoosein and Lewin (12). This antibody does not react with the product of the *CIT2* gene (14). For immunoblots, 25 μl from each fraction was dissociated in electrophoresis sample buffer and were separated on 10% sodium dodecyl sulfate-polyacrylamide gels (7). Proteins were transferred to nitrocellulose sheets and reacted with rabbit antibody according to the method of Tobin et al. (26). Antibody-antigen complexes were detected by using ^{125}I-labeled protein A.

Cytology. Fractions from the Nycodenz gradients were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) overnight and were then pelleted at 45,000 rpm in a Beckman Ti50 rotor for 30 min. The pellets were postfixed with 1% OsO_4 in 0.1 M cacodylate buffer for 1 h, stained with uranyl acetate for 1 h, dehydrated, and embedded in Epon 812. Thin sections were examined in a Jeol JEM 1000XII electron microscope.

Materials. Nycodenz and fine chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) or from Boehringer-Mannheim Biochemicals (Indianapolis, Ind.). Dehydrated culture media came from Difco Laboratories (Detroit, Mich.). Zymolyase 20T was purchased from ICN Biochemicals, Inc. (Irvine, Calif.). Materials for gel electrophoresis were obtained from Gallard and Schlesinger, Inc. (Carle Place, N.Y.).

RESULTS AND DISCUSSION

Distribution of citrate synthase in wild-type cells. To determine whether extramitochondrial citrate synthase cofractionated with peroxisomes, we induced peroxisome formation by growing cells in oleate-containing medium. A crude organelle pellet was isolated from homogenated spheroplasts by centrifugation at 27,000 × *g*. This pellet contained more than 85% of the citrate synthase activity (52.4 U of 61.6 U total) and more than 90% of the fumarase activity (373.8 U of 402.4 U), so that most of soluble mitochondrial enzymes (and virtually all of the cytochrome *c* oxidase) were recovered in this pellet. In addition, more than 99% of the acyl-CoA oxidase, a marker enzyme for the peroxisomal matrix, was associated with the high-speed pellet.

The resuspended pellet was fractionated on a Nycodenz stepped gradient. Protein concentrations and activities of acyl-CoA oxidase, citrate synthase, cytochrome *c* oxidase, catalase, and malate dehydrogenase were determined for each fraction. Fumarase could not be assayed in the gradient fractions because of the A_{240} of Nycodenz. The activity profiles for strain GRF18, which has wild-type alleles for *CIT1* and *CIT2*, are shown in Fig. 1. Much of the activity was found at the top of the gradient, but within the gradient, citrate synthase was distributed biomodally: one peak cosedimented with mitochondria (as identified by the marker enzymes cytochrome *c* oxidase and malate dehydrogenase), and the other coincided with peroxisomes (as detected by acyl-CoA oxidase and catalase activities). The peroxisomal peak was not completely uniform, judging from the fact that the peak of catalase activity (fraction 11) did not coincide with that of acyl-CoA oxidase and the peak of citrate synthase (fraction 10).

The malate dehydrogenase profile (Fig. 1A) and electron micrographs (see below) indicated that the peroxisomal fraction was contaminated with a small amount of mitochondria. Alternatively, malate dehydrogenase, which participates in the glyoxylate cycle, may have a peroxisomal

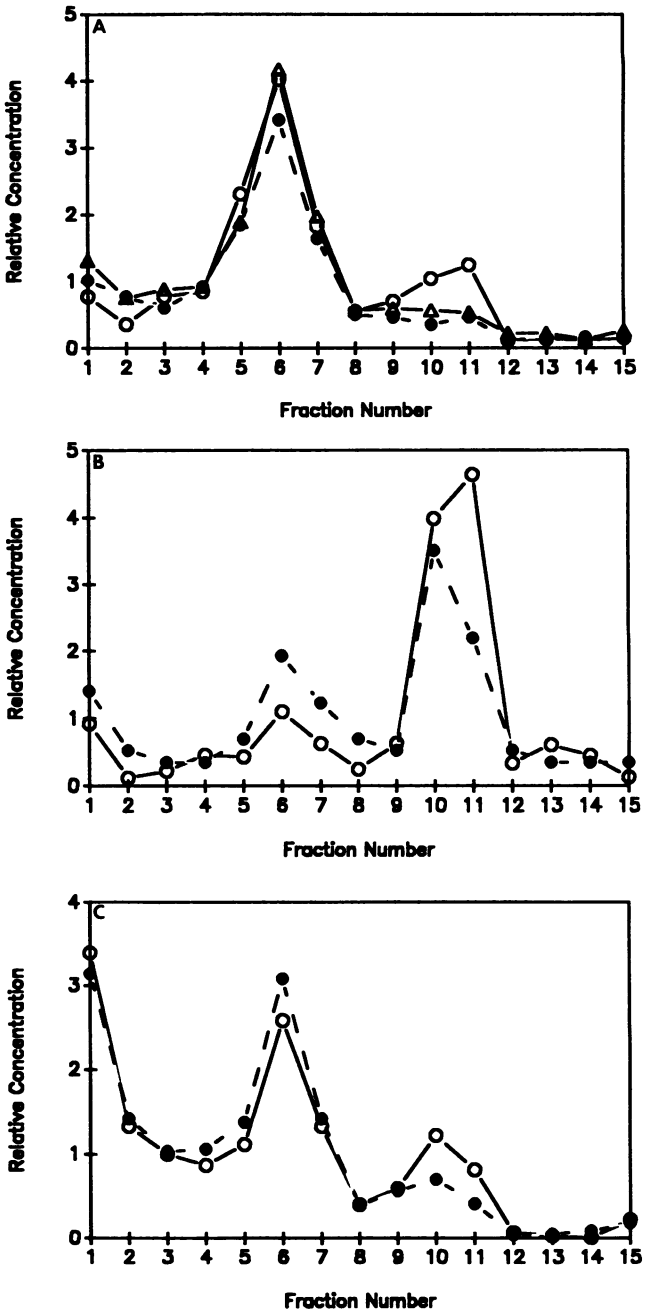

FIG. 1. Citrate synthase activity associated with peroxisomes in wild-type yeast cells. A membrane fraction containing mitochondria and peroxisomes from a strain with wild-type alleles at *CIT1* and *CIT2* was separated by sedimentation in a Nycodenz step gradient. Gradients were siphoned from the top, so that fraction 1 was the top of the gradient and fraction 15 was the bottom. (A) Relative concentrations of protein (△), malate dehydrogenase (○), and cytochrome *c* oxidase (●). (B) Relative concentrations of catalase (○) and acyl-CoA oxidase (●). (C) Relative concentrations of citrate synthase with (●) and without (○) pH 8.1 pretreatment.

isozyme. This seems to be the case in leaf glyoxysomes (27). Consequently, we wanted to determine whether the citrate synthase recovered with peroxisomes was actually the mitochondrial isozyme. Extramitochondrial citrate synthase is inactivated by incubation at pH 8.1, but the mitochondrial enzyme is stable at this pH (21). After preincubation at pH

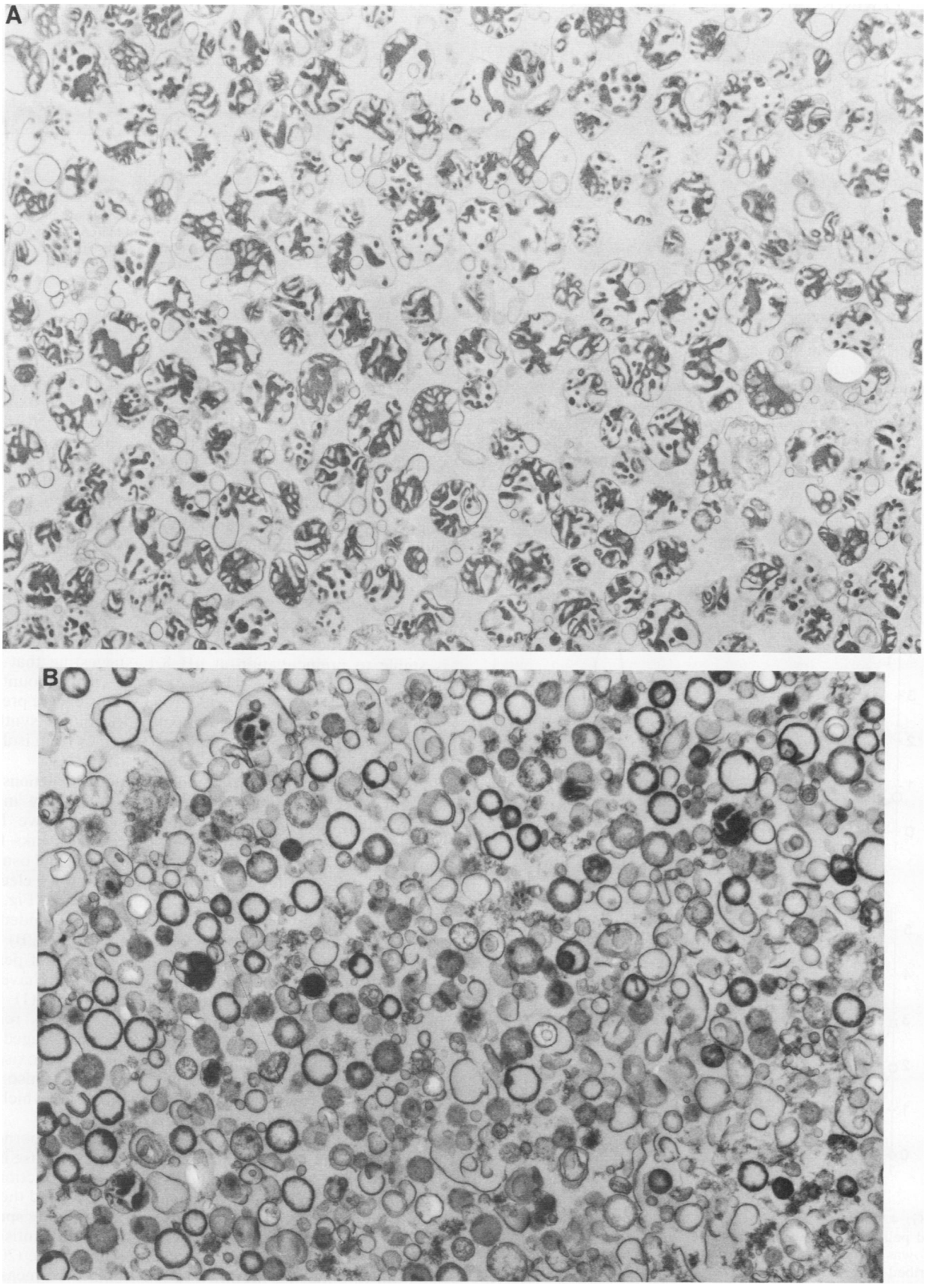

FIG. 2. Mitochondrial and peroxisomal fractions from the Nycodenz gradients. Immediately upon fractionation of the gradient represented in Fig. 1, samples were taken from those fractions in which, on the basis of previous experiments, mitochondria and peroxisomes were thought to band. These samples were fixed overnight with glutaraldehyde and prepared for microscopy as described in Materials and Methods. (A) Fraction 6; (B) fraction 10. Magnification, ×7,840.

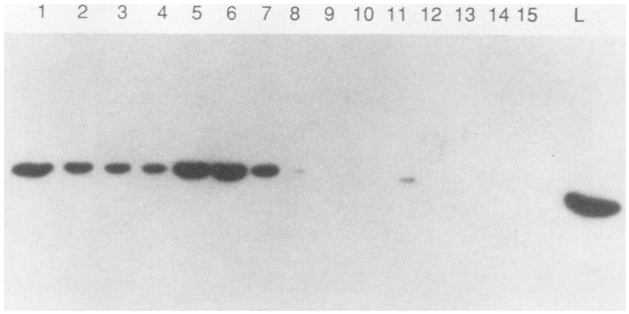

FIG. 3. Immunoreactive citrate synthase in fractions from the density gradient. Samples (25 μl) from each fraction the gradient represented in Fig. 1 were separated by electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose membranes and reacted with antiserum specific for mitochondrial citrate synthase. The position of immune complexes was detected by incubation with ^{125}I-labeled protein A. Lane L, reaction with 2 μl (19 μl) of the material loaded on the gradient.

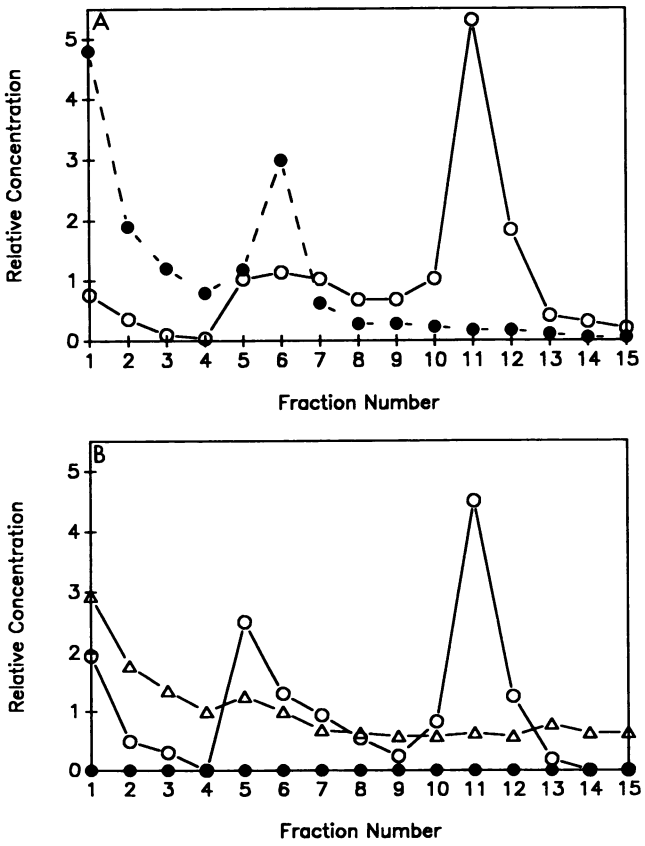

FIG. 4. Distribution of citrate synthase in *cit1* cells. A high-speed pellet containing mitochondria and peroxisomes from strain GK1 was prepared and fractionated on Nycodenz gradients as described in the legend to Fig. 1. Enzyme activities are expressed as relative concentrations. (A) Distributions of catalase (○) and malate synthase (●). (B) Distributions of protein (△) and citrate synthase without pH 8.1 pretreatment (○). No activity was detected in any of the fractions after incubation at pH 8.1 (●).

TABLE 1. Specific enzymatic activities of the organelle fraction[a]

Genotype	Activity (U/mg of protein)		
	Catalase[b]	Citrate synthase[c]	Cytochrome oxidase[d]
CIT1 CIT2	4.2	0.80	16.2
cit1 CIT2	4.3	0.08	5.6
CIT1 cit2	1.1	0.81	9.7

[a] Cells were grown overnight in oleate induction medium, and the high-speed membrane pellet was prepared as described in Materials and Methods. Assays were performed in duplicate or triplicate, and results varied less than 15% for catalase and citrate synthase and less than 20% for cytochrome oxidase.
[b] Calculated as determined by Baudhuin (2).
[c] Units are micromoles of reduced CoA formed per minute.
[d] Units are moles (10^8) of cytochrome oxidized per minute.

8.1, the citrate synthase activity recovered in the mitochondrial peak rose marginally, whereas that in the peroxisome peak diminished (Fig. 1C). The specific citrate synthase activity of the peroxisomal fraction decreased from 0.55 to 0.40 U/mg of protein, but the specific activity of the mitochondrial fraction increased after incubation at pH 8.1. In this experiment, fraction 6 increased from 0.37 to 0.64 U/mg of protein, for example. This increase would elevate mitochondrial activity contaminating the peroxisomal peak and explains why citrate synthase activity was not totally eliminated from this peak by pH 8.1 treatment.

These results suggest that most of the citrate synthase activity in the peroxisomal fraction is the extramitochondrial isozyme. The activity at the top of the gradient, in contrast, is stable to preincubation at pH 8.1, suggesting that it is derived from mitochondria. Since only small amounts of malate dehydrogenase and cytochrome oxidase were present in this fraction, it appears that mitochondrial citrate synthase was released more readily than these enzymes from isolated mitochondria.

Purity of the mitochondrial and peroxisomal fractions. To account for the residual citrate synthase activity in the peroxisomal fraction after alkaline incubation, we fixed samples of the mitochondrial and peroxisomal peaks from the gradient shown in Fig. 1 with glutaraldehyde and osmium tetroxide and embedded them for examination by electron microscopy (Fig. 2). The sample from fraction 6 (Fig. 2A) showed a relatively homogeneous collection of condensed-form mitochondria. In contrast, fraction 10 (Fig. 2B) contained smaller, single-membrane vesicles typical of peroxisomes. Although some of the vesicles appeared to have lost their electron-opaque contents, Alexson et al. (1) have demonstrated that many of the matrix enzymes remain associated with peroxisomes that have been damaged, accounting for the fact that we detected little peroxisomal activity at the top of the gradient. This fraction also contained a number of small, dense mitochondria, which explains the contaminating pH 8.1-resistant enzyme.

That the alkali-stable enzyme is mitochondrial is borne out by the distribution of immunoreactive citrate synthase in the gradients. We separated samples from each fraction on sodium dodecyl sulfate-polyacrylamide gels, blotted the gels to nitrocellulose, and reacted them with an antibody specific for mitochondrial citrate synthase (Fig. 3). This antiserum does not react with the extramitochondrial isozyme (20). In addition to exposing the filter to X-ray film, we measured radioactivity directly for 5 h, using an Ambis radioanalytic imaging system.

Our results suggested that there was a minor contamina-

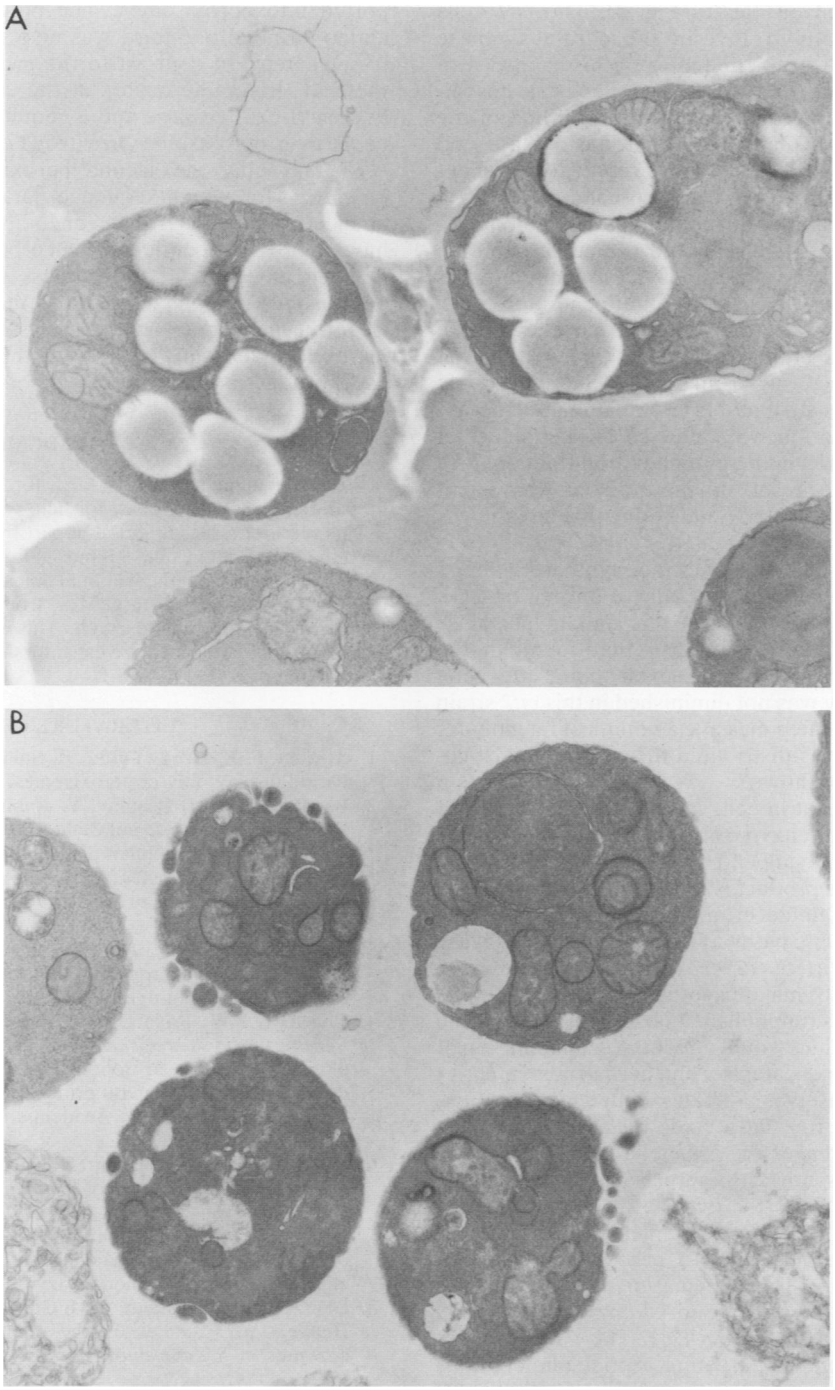

FIG. 5. Accumulation of fat-containing vesicles by a strain bearing a *CIT2* disruption. Cells containing a disruption of the *CIT2* gene (A) and cells containing wild-type *CIT1* and *CIT2* alleles (B) were grown overnight on induction medium and converted to spheroplasts by using Zymolyase 20T. They were fixed in glutaraldehyde and prepared for microscopy as described in Materials and Methods. Magnification, ×17,000.

tion of mitochondrial citrate synthase in the peroxisome fraction: in the samples from wild-type cells, there was substantial reaction at the top of the gradient (110.8 ± 3.5 cpm; 16.1 cpm/μg of protein). The peak antibody binding within the gradient was observed in mitochondrial fractions 5 and 6 (143.3 ± 4 cpm; 10.4 cpm/μg). A minor reaction was observed in peroxisomal fraction 11 (10.2 ± 2.2 cpm; 2.5 cpm/μg). In the gradient from *cit1* cells, no immune reaction was detected (data not shown). Using a polyclonal antiserum specific for peroxisomal proteins from *Candida tropicalis* (23), we determined that the major cross-reacting protein (probably catalase) cofractionated with the peak catalase activity, as expected, but that some antibody binding was detected in the mitochondrial peak (data not shown).

Enzyme distribution in a *cit1* deletion. These results were substantiated by the enzyme profiles of subcellular fractions

of a *cit1* strain induced on oleate (Fig. 4). The *cit1* cells contained only about 10 to 15% of the citrate synthase activity of the wild-type strain, and most of this banded with the peroxisome fraction (Fig. 4B). A smaller peak cosedimented with the mitochondria. A greater proportion of the mitochondrial enzymes (such as cytochrome oxidase and malate dehydrogenase) remained at the top of the gradient than with the lysate from *CIT1* cells, perhaps indicating a decreased density or stability of the mitochondria from this strain. Krispal et al. (15) have noted a decrease in the activity of several matrix enzymes in a *cit1* mutant, and this decrease might affect the density of the mitochondria.

The specific activity of the peroxisomal citrate synthase was much higher than that associated with mitochondria (0.39 versus 0.094 U/mg), suggesting that the activity in the mitochondrial peak was attributable to contaminating peroxisomes. This contamination was reflected by a shoulder of catalase activity (Fig. 4A) in the mitochondrial fractions. All of the citrate synthase present in the gradient was inactivated by preincubation at pH 8.1, indicating that all of it was the extramitochondrial form (Fig. 4B).

To determine the distribution of citrate synthase in a *cit2* mutant, we grew the cells in oleate, but we did not observe normal induction of peroxisomes in this mutant. Catalase activity was approximately one-fourth that found in the wild-type cells or in the *cit1* strain, even though the total citrate synthase activity was not diminished in this *cit2* strain (Table 1). Cells accumulated massive amounts of fat globules (Fig. 5), and catalase was distributed throughout the Nycodenz gradient (data not shown).

The accumulation of fat in cells lacking the *CIT2* product seems to implicate this enzyme in the catabolism of fatty acids. Since the activity appeared to be peroxisomal, we infer that the *CIT2* gene product is the citrate synthase of the glyoxylate pathway. Duntze et al. have demonstrated the existence of the glyoxylate pathway enzymes isocitrate lyase and malate synthase in *S. cerevisiae* (8), and we have observed the presence of malate synthase in the peroxisome fraction of induced cells (unpublished observation). Why the absence of the *cit2* product would affect the sedimentation of peroxisomal enzymes like catalase, and perhaps the integrity of the organelles themselves, remains under investigation. One possibility is that free fatty acids released during disruption of the cells damages the organelle membranes.

Targeting of citrate synthase to peroxisomes. Peroxisomal enzymes are imported from the cytoplasm into preexisting organelles after their synthesis is completed (16). The peptide sequences that target them to peroxisomes are not removed after transport. The enzyme encoded by *CIT2* is 75% homologous with the mitochondrial enzyme but differs at the amino and carboxyl termini (21). The *CIT2* product lacks a typical mitochondrial targeting peptide and contains SKL as a carboxyl-terminal tripeptide. Gould et al. (10, 11) have demonstrated that SKL, which occurs at the carboxyl termini of several peroxisomal proteins, is one type of signal that can act to target proteins to mammalian peroxisomes in vivo.

Import experiments have been carried out in vitro with peroxisomes from the yeast *C. tropicalis*. Acyl-CoA oxidase, the first enzyme in the peroxisomal β-oxidation cycle, was shown to contain two regions that are individually capable of targeting proteins to peroxisomes (24). Similar conclusions were drawn from experiments in vivo in which the *POX4* gene from *C. tropicalis* was expressed in *Candida maltosa* (13). In these experiments, a peptide encoded by the carboxyl-terminal two-thirds of *POX4* was efficiently transported into peroxisomes. The polypeptide containing the amino-terminal one-third was also imported, although to a lesser extent. In contrast to the protein from *C. tropicalis*, the SKL tripeptide occurs at the carboxyl terminus of rat liver acyl-CoA oxidase and is required to target this protein to rat liver peroxisomes in vitro (17).

CIT2 is only the second peroxisomal protein from *S. cerevisiae* for which sequence data are available. The sequence of the *CTA1* gene, encoding catalase A, does not have the SKL tripeptide at the carboxyl terminus (4). Thus, it would appear that the process of targeting proteins to peroxisomes is complex and, as indicated by Gould et al. (10), that more than one type of signal must exist. Experiments to investigate the role of the SKL tripeptide in the *CIT2* product are in progress.

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