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Absence of DNA in Peroxisomes of *Candida tropicalis*

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Yeast peroxisomes were purified to near homogeneity from cells of *Candida tropicalis* grown on oleic acid for the purpose of examining the possible presence of DNA in this organelle. The purification procedure includes the effective conversion of cells to spheroplasts with Zymolyase and sodium sulfite and the separation of the organelles at extremely low ionic strength. The mitochondrial contamination was less than 1%, based on several criteria, and the yield of peroxisomes was about 40%. The purified peroxisomal fraction contained a very small amount of DNA, which yielded restriction fragments indistinguishable from those of mitochondrial DNA. The absence of DNA in peroxisomes was also supported by cesium chloride density gradient centrifugation of the organelles lysed with a detergent, staining of the organelles with a fluorescent dye specific to DNA, and labeling of the DNA with [^{3}H]adenine.

We have previously shown that there are two functionally distinct long-chain acyl coenzyme A (acyl-CoA) synthetases (EC 6.2.1.3) in a yeast, *Candida lipolytica* (3, 11). Acyl-CoA synthetase I is responsible for the synthesis of cellular lipids, whereas acyl-CoA synthetase II participates in the degradation of fatty acids (3, 4). Consistent with their functions is the fact that the former is distributed among various subcellular fractions where glycerophosphate acyltransferase is located, while the latter is localized in peroxisomes (microbodies) where the acyl-CoA oxidizing system is located (10). Studies with mutant strains defective in acyl-CoA synthetase II have suggested that the functional expression of the enzyme in peroxisomes is regulated by some proteins which might be encoded extrachromosomally (T. Kamiryo, unpublished data). The possible participation of extrachromosomal genes has not been examined by genetic means because of the lack of mating ability in the yeast strains used. In this regard, special attention was paid to reports (12, 13) that suggested the presence of a DNA in the peroxisomes of a related yeast, *Candida tropicalis*. The DNA was proposed to be a double-stranded linear molecule with a contour length of about 12 μm (12; M. Osumi, personal communication) and a buoyant density of 1.693 g/ml (13). This putative peroxisomal DNA may have a role in the proper expression of acyl-CoA synthetase II.

To gain insight into the regulatory mechanism of the expression of peroxisomal enzymes, we attempted to verify the presence of the DNA in peroxisomes and to characterize it biochemically. For this purpose, it is of crucial importance to use highly purified peroxisomes that have been freed of mitochondria, which contain DNA with a buoyant density (1.691 g/ml) very similar to that of the putative peroxisomal DNA (13). Since a suitable peroxisomal preparation has not been reported, we purified yeast peroxisomes to near homogeneity. The present report describes a procedure for preparing peroxisomes with a purity of more than 99%. This peroxisomal preparation provided no evidence for the presence of a specific DNA in spite of our extensive search for it.

MATERIALS AND METHODS

Enzymes and chemicals. Zymolyase 60000 was obtained from Kirin Brewery Co., Tokyo, Japan; restriction endonucleases were from Takara Shuzo Co., Kyoto, Japan; proteinase K was from E. Merck AG, Darmstadt, Germany. RNase T_1 (grade IV), RNase A (type I-A), cytochrome *c* (type III), and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, Mo. RNA polymerase from *Escherichia coli* was prepared by the method of Burgess and Jendrisak (1). Yeast extract and peptone were products of Difco Laboratories, Detroit, Mich.; Ficoll 400 and Percoll were from Pharmacia Fine Chemicals, Uppsala, Sweden; Brij 58 was from Kao-Atlas, Tokyo, Japan. [8-^{3}H]adenine was provided by The Radiochemical Centre, Amersham, England; Triton X-100, Univer-Gel, and Agarose LE were manufactured by Nakarai Chemicals, Kyoto, Japan. 4′,6-Diamidino-2-phenylindole (DAPI) and ethidium bromide were obtained from Aldrich Chemical Co., Milwaukee, Wis.; Spurr's resin was from Taab Laboratories, Reading, England. Other chemicals were of highest commercial purity.

Yeast strain and culture. *Candida tropicalis* pK233 was used throughout the study because of its high level of proliferating peroxisomes (14). Cells were grown at 30°C aerobically in YPBO medium composed of 0.3% yeast extract, 0.5% peptone, 0.5% K_2HPO_4, 0.5% KH_2PO_4, 1% Brij 58, and 1% (wt/vol) oleic acid. The control cells, without the proliferation of peroxisomes, were grown in YPBG medium, which was similar to YPBO medium except that the oleic acid was replaced by 2% glucose.

Preparation of particulate fraction. Forty grams (wet weight) of cells, harvested at late-logarithmic phase (about 3×10^7 cells per ml), was washed with water and suspended in 160 ml of 0.5 M KCl containing 5 mM potassium 3-(*N*-morpholino)propanesulfonate (MOPS) (pH 7.2) and 10 mM Na_2SO_3. The cells were converted to spheroplasts by incubation with 40 mg of Zymolyase 60000 at 30°C for 40 to 60 min. All subsequent operations, including the separation of peroxisomes, were conducted at 0 to 4°C. The spheroplasts, collected by centrifugation at 2,500 × *g* for 8 min, were broken in 120 ml of F buffer, which contained 5% Ficoll 400, 0.6 M sorbitol, 2.5 mM MOPS (pH 7.2), and 1 mM EDTA, with a Teflon homogenizer by 10 down-and-up strokes. The homogenate was centrifuged at 1,000 × *g* for 10 min. The supernatant was saved, and the precipitate was homogenized again in 60 ml of F buffer by three down-and-up strokes. It was centrifuged as above, and the combined supernatant was further centrifuged at 20,000 × *g* for 20 min. The precipitated particulate fraction was suspended in about 40 ml of F buffer.

Sucrose density gradient centrifugation. About 6 ml of the particulate fraction was applied onto a discontinuous gradient consisting of 4-, 6-, 12-, and 6-ml sucrose solutions of 25, 35, 42, and 53% (wt/wt), respectively; all sucrose solutions contained 2.5 mM MOPS (pH 7.2) and 0.5 mM EDTA. The tubes were centrifuged at 101,000 × *g* for 1 h with an RPV50T rotor (Hitachi, Tokyo, Japan) and fractionated from the bottom. When a peroxisomal pellet was needed, fractions enriched with the organelle were combined, dialyzed against 100 volumes of 0.5 M sucrose containing 5 mM MOPS (pH 7.2) and 3 mM $CaCl_2$ for 1 h, and then centrifuged at 20,000 × *g* for 10 min.

Percoll density gradient centrifugation. About 6 ml of the particulate fraction was layered on top of 28 ml of a solution of Percoll in F buffer (adjusted to a density of 1.070 g/ml with Percoll). The tubes were centrifuged at 65,000 × *g* for 40 min with an RPV50T rotor and fractionated from the bottom. When it was necessary to remove the Percoll, the fraction was diluted with 3 volumes of F buffer and centrifuged at 20,000 × *g* for 10 min. The precipitate was washed two more times with F buffer.

Assay of enzymes. Activities of catalase (EC 1.11.1.6) and cytochrome *c* oxidase (EC 1.9.3.1) were assayed at 25°C spectrophotometrically by following the consumption of the substrates. The reaction mixture for the assay of catalase contained 50 mM potassium phosphate (pH 7.2) and 11 mM H_2O_2 (17), and that for the assay of cytochrome *c* oxidase contained 100 mM potassium phosphate (pH 7.2), 1 mM EDTA, and 28 μM cytochrome *c* reduced with ascorbic acid (15). The preparations to be assayed were diluted with F buffer to an appropriate extent and mixed with 1 volume of 50 mM potassium phosphate (pH 7.2) containing 1% (wt/vol) Triton X-100. One enzyme unit is defined as the amount which catalyzes the conversion of 1 μmol of substrate per min. Protein was determined by the method of Lowry et al. (9) with bovine serum albumin as the standard.

Preparation of DNA. Purified organelles were disrupted with sodium dodecyl sulfate and proteinase K. DNA was isolated by the conventional procedure (2), including phenol extraction, ethanol precipitation, and digestion with a combination of RNase T_1 and RNase A, followed by phenol extraction and ethanol precipitation.

Equilibrium centrifugation of lysed organelles. Organelles in 0.5 ml of sucrose solution were mixed with 0.5 ml of a solution composed of 1% sodium *N*-lauroyl sarcosine, 80% (vol/vol) glycerol, 2 mM EDTA, and 15 μM ethidium bromide. The mixture was added to 3.9 ml of CsCl solution (1.800 g/ml) containing 1 mM EDTA and 7.5 μM ethidium bromide and centrifuged at 220,800 × *g* and 15°C for 14 h with an RPV65T rotor (Hitachi).

Incorporation of [^{3}H]adenine. Cells were grown in 150 ml of the YPBO medium supplemented with 90 μCi of [8-^{3}H]adenine (23 Ci/mmol). The particulate fraction derived from the labeled cells was subjected to sucrose density gradient centrifugation. A 1-ml portion of each fraction (2.5 ml) was acidified with 13% trichloroacetic acid, and the precipitate was collected on an FC-50 glass filter (Toyo Roshi, Tokyo, Japan). The radioactivity was counted in Univer-Gel with a liquid scintillation spectrometer. Nucleic acids isolated from another portion of 1 ml were digested with the combination of RNase T_1 and RNase A, and the acid-insoluble radioactivity was counted as described above.

Staining of organelles. Organelles in sucrose solution were stained with 0.4 μM DAPI by the method of Kuroiwa and Suzuki (8) and examined under a BHS-RF-A epifluorescence microscope (Olympus, Tokyo, Japan) with a U-excitation unit (360 nm) and a 495-nm barrier filter. They were also stained with 90 μM ethidium bromide and examined with a B-excitation unit (400 to 490 nm) and a 610-nm barrier filter.

Electron microscopy. Organelles in sucrose solution were fixed with 5% glutaraldehyde, postfixed with 1% osmium tetraoxide, and enbedded in Spurr's resin with the aid of 2% agar. The procedure was essentially according to Shigenaka et al. (18). The ultrathin sections prepared were viewed with a JEM 100S electron microscope (JEOL, Tokyo, Japan) at 100 kV.

RESULTS AND DISCUSSION

Purification of peroxisomes. Table 1 summarizes the result of a typical purification of peroxisomes. Catalase was used as the marker enzyme of peroxisomes, and cytochrome *c* oxidase was used for mitochondria (5). Cells were converted to spheroplasts with Zymolyase in the presence of sodium sulfite (6) instead of 2-mercaptoethanol or dithiothreitol. The sulfite at a low concentration increased the susceptibility of cells to the enzyme more effectively than the thiol compounds which have been used by many workers. This was evident from the fact that sulfite yielded a higher catalase activity in the particulate

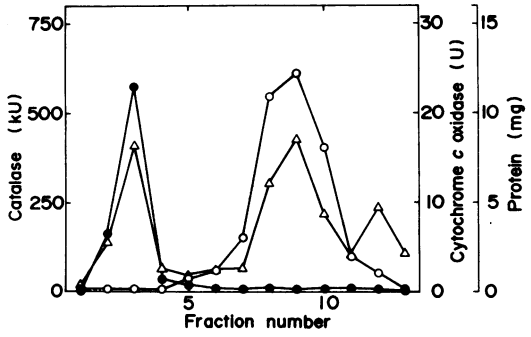

FIG. 1. Separation of peroxisomes by sucrose density gradient centrifugation. Cells were grown in YPBO medium. The particulate fraction derived from about 5 g of wet cells was applied to a discontinuous sucrose density gradient. Symbols: ●, catalase activity; ○, cytochrome *c* oxidase activity; △, protein.

fraction (about 50% of the activity in the homogenate) than 2-mercaptoethanol did (at most 10%).

Together with the use of a vertical rotor, the buffer with an extremely low ionic strength facilitated the efficient separation of peroxisomes and mitochondria. One run of centrifugation with eight tubes was sufficient for the amount of particulate fraction (about 1 g as protein) derived from 80 g of wet cells. Figure 1 shows a result of the separation by sucrose density gradient centrifugation. More than 80% of catalase activity in the particulate fraction was recovered in the peroxisomal fraction, whereas only 0.3% of cytochrome *c* oxidase activity was found in this fraction (Table 1). The peroxisomal fraction thus obtained exhibited a markedly high catalase/oxidase ratio (3,370, expressed as catalase activity in enzyme units × 10^3, divided by cytochrome *c* oxidase activity in enzyme units) and a high specific activity of catalase (85 kU/mg of protein). Mitochondria in the peroxisomal fraction were estimated to be less than 1% from the observations that mitochondria were the major contaminant in the fraction and that they were at most twice as abundant as peroxisomes in cells (see below).

The cells grown on glucose contained many times fewer peroxisomes. This preparation had a catalase specific activity of about 0.4 kU per mg of protein and a catalase/oxidase ratio of about 1.2.

When the peroxisomes obtained from the sucrose density gradient were diluted with F buffer to reduce the sucrose concentration, they lost a considerable amount of catalase activity due to breakage by osmotic shock. To prevent the breakage, the preparation was dialyzed against 0.5 M sucrose containing 3 mM $CaCl_2$ (or $MgCl_2$) before centrifugation to collect the organelle. The peroxisomes thus obtained retained essentially all the catalase activity and were used for isolating DNA (see below). Although the peroxisomes obtained from the Percoll density gradient resisted such dilution, their catalase/oxidase ratio was lower (80 to 140) than that of the peroxisomes from the sucrose density gradient. Therefore, peroxisomes used in the present study were prepared by sucrose density gradient centrifugation unless otherwise specified.

In sucrose density gradient centrifugation, peroxisomes exhibited a buoyant density (about 1.24 g/ml) higher than mitochondria. On the other hand, the density of peroxisomes was 1.065 ± 0.001 g/ml and that of mitochondria was 1.078 ± 0.001 g/ml in Percoll solution.

Purity of peroxisomes. The purity of the peroxisomal preparation was examined with respect to the contamination of mitochondria, because they contained DNA with a buoyant density similar to that of the putative peroxisomal DNA. Peroxisomes were identified by electron microscopy (data not shown). The major contaminant of the peroxisomal fraction was mitochondria. Counting of organelles on electron micrographs revealed that the impurity, consisting of mitochondria and other unidentified structures, was about 0.5%.

To confirm this value, the polypeptides of both the organelles were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Figure 2 shows the profiles of polypeptides derived from peroxisomes (lane 2) and from mitochondria (lane 6). The relative amounts of organelles analyzed were adjusted to

TABLE 1. Purification of peroxisomes from oleic acid-grown cells[a]

Fraction	Protein (mg)	Catalase		Cytochrome *c* oxidase		Catalase/ oxidase ratio[b]
		Total (kU)	Sp act (kU/mg)	Total (U)	Sp act (U/mg)	
Homogenate	2,805	20,642	7.4	1,622	0.58	12.7
Particulate	402	9,165	23	700	1.7	13.1
Peroxisomes	93	7,929	85	2.35	0.025	3,370
Mitochondria	160	191	1.2	536	3.4	0.36

[a] A 41-g (wet weight) sample of freshly grown cells was used.
[b] Catalase activity in enzyme units × 10^3 (kU) was divided by cytochrome *c* oxidase activity in enzyme units (U).

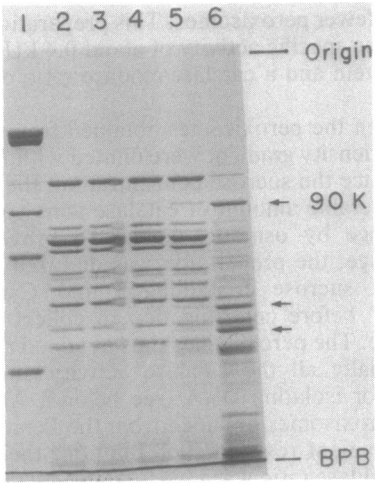

FIG. 2. Slab gel electrophoresis of polypeptides from organelles. Electrophoresis on a sodium dodecyl sulfate-polyacrylamide slab gel containing 8% acrylamide was done by the method of King and Laemmli (8). Bromophenol blue (BPB) served as the tracking dye. The gel was stained with 0.4% Coomassie brilliant blue dissolved in ethanol-acetic acid-water (50:10:40, vol/vol) and was destained with ethanol-acetic acid-water (5:7.5:87.5, vol/vol). Lane 1, Marker peptides (subunits of *E. coli* RNA polymerase and bovine serum albumin); lane 2, 12 μg as protein of peroxisomes; lane 3, 12 μg of peroxisomes plus 0.02 μg of mitochondria; lane 4, 12 μg of peroxisomes plus 0.2 μg of mitochondria; lane 5, 12 μg of peroxisomes plus 2 μg of mitochondria; lane 6, 20 μg of mitochondria. Gel strips of similar experiments were scanned with an FD-A III densitometer (Fuji Riken) at 570 nm.

the expected ratio of their intracellular contents. Polypeptides marked with arrows were specific to mitochondria and were used to estimate the contamination of mitochondria in the peroxisomal preparation. For this purpose, peroxisomes were mixed with 0.1% (Fig. 2, lane 3), 1% (lane 4), and 10% (lane 5) of the amount of mitochondria applied on lane 6. Scanning of the stained band of 90,000 daltons indicated that the extent of the contamination was about 0.3%; this value was consistent with the electron microscopic observation.

From these results, and from the activities of the marker enzymes as well, we concluded that the purity of the peroxisomal preparation was more than 99%. This purity is much higher than those hitherto reported for yeast and animal equivalents (5, 7, 10). The procedure described here would be applicable to purification not only of peroxisomes, but also of mitochondria from various eucaryotic cells.

DNA from peroxisomal preparation. We attempted to isolate DNA from the purified peroxisomes. To minimize their damage, the fraction from the sucrose density gradient was dialyzed before the particles were collected. A very small amount of DNA was obtained. The use of carrier RNA in the ethanol precipitation did not affect the yield. Figure 3 shows fragments generated by the digestion of the DNA with restriction endonuclease *Eco*RI or *Hae*III. In this experiment the peroxisomal DNA preparation was derived from 10 times more cells than the mitochondrial DNA. Extremely faint bands observed for the DNA from the peroxisomal fraction (Fig. 3, lane 3 and 5) were indistinguishable from those for the mitochondrial DNA (lane 2 and 4). Digestion with endonuclease *Bam*HI or *Hin*dIII showed a similar result. Thus, the small amount of DNA obtained could be attributed to contamination with mitochondria. Furthermore, no specific DNA was obtained from the peroxi-

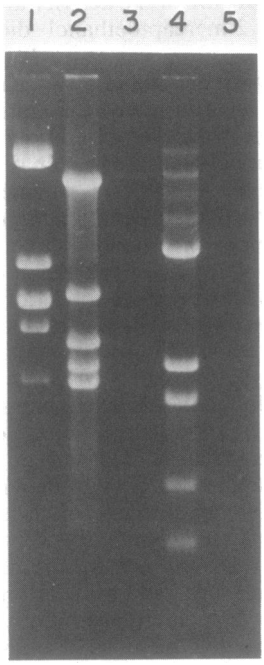

FIG. 3. Restriction fragments of DNA from organelles. Peroxisomes and mitochondria were prepared from 62 g of wet cells grown in YPBO medium. DNA isolated from them was dissolved in water: 0.06 ml for the preparation from peroxisomes and 0.6 ml for that from mitochondria. DNA in 10 μl of solution was digested with restriction endonuclease under the conditions recommended by the vendor. DNA fragments were separated by electrophoresis on 0.5% agarose gels and stained with ethidium bromide (16). Lane 1, Marker fragments (lambda phage DNA digested with *Eco*RI); lane 2, mitochondrial DNA digested with *Eco*RI; lane 3, DNA from peroxisomal fraction, digested with *Eco*RI; lane 4, mitochondrial DNA digested with *Hae*III; lane 5, DNA from peroxisomal fraction, digested with *Hae*III.

somes prepared by Percoll density gradient centrifugation.

It is worth noting that the mitochondrial DNA of *C. tropicalis* was estimated to be about 35 kilobase pairs long from the lengths of the restriction fragments; this is very close in size to the putative peroxisomal DNA (12; M. Osumi, personal communication). Consideration of the size (14; our data not shown) and the packed volume of the organelles, together with the electron microscopic view of the cells proliferating peroxisomes (10, 14), suggested that the number of peroxisomal particles in cells was more than one half of that of mitochondria. The peroxisomal fraction should contain, therefore, at least one-half of the amount of DNA isolated from the mitochondrial fraction, but in fact the amount actually obtained was markedly smaller. One could speculate that only constitutive peroxisomes contain DNA, whereas those induced by fatty acid do not. However, this was not the case, because no DNA was isolated from the peroxisomes of cells grown on glucose.

Staining of DNA without isolation. The results described above suggested the absence of DNA in peroxisomes. The possibility was not excluded, however, that the peroxisomal DNA was specifically lost during the process of preparation. Therefore, peroxisomes obtained from the sucrose density gradient were immediately lysed with sodium lauroyl sarcosine and subjected to CsCl density gradient centrifugation in the presence of ethidium bromide. In contrast to the mitochondrial preparation, the peroxisomal preparation exhibited no nucleic acid throughout the tube (data not shown). The staining of the gradient with DAPI, a fluorescent dye that preferentially binds to DNA rich in adenine-thymine base pairs (19), also gave no sign of DNA (data not shown).

Furthermore, we stained the organelles directly with either ethidium bromide or DAPI and observed them with a fluorescence microscope. Mitochondria were clearly stained with both fluorescent dyes, whereas peroxisomes were not stained with either dye (data not shown). Although these observations were consistent with the absence of DNA in peroxisomes, they might result from the low affinity of the peroxisomal DNA to the fluorescent dyes. The next experiment was designed to detect the DNA without the use of dye.

Incorporation of [^{3}H]adenine into peroxisomes. Since the putative peroxisomal DNA was reported to have a low buoyant density (13), we attempted to label it with [^{3}H]adenine. The particulate fraction was prepared from cells grown in the presence of [8-^{3}H]adenine and subjected to sucrose density gradient centrifugation. The RNase-resistant radioactivity in the resulting peroxisomal fraction was 2.6% of that in the mitochondrial fraction and was about threefold higher than what was expected from the distribution of cytochrome *c* oxidase activity (data not shown). Nevertheless, it was too small to indicate the putative peroxisomal DNA, which should be one-half of the amount of mitochondrial DNA as discussed above.

To our knowledge, the present investigation is the first extensive search for DNA specific for peroxisomes. Although the experiments described here do not exclude the possibility that peroxisomes contain DNA with unusual characteristics, all the results obtained are consistent with the absence of DNA in this organelle. In this context, it should be noted that the peroxisomal DNA would contain few or no ribonucleotides, because the omission of RNase digestion did not affect the yield of DNA. The substance of the putative peroxisomal DNA is presumed to be the mitochondrial DNA from the following considerations: first, its size (12) is essentially the same as that of the mitochondrial DNA; second, the small difference of buoyant density in CsCl reported between them (13) could be explained by the elimination of regions rich in adenine-thymidine base pairs. Thus, we conclude that DNA is absent in peroxisomes of oleic acid-grown cells of *C. tropicalis*. The expression of peroxisomal enzymes, therefore, must be under the control of nuclear genes.

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