MOLECULAR AND CELLULAR BIOLOGY, May 1987, p. 1848–1855
0270-7306/87/051848-08$02.00/0

Vol. 7, No. 5

Efficient Association of In Vitro Translation Products with Purified, Stable *Candida tropicalis* Peroxisomes

GILLIAN M. SMALL,* TSUNEO IMANAKA, HELEN SHIO, AND PAUL B. LAZAROW
The Rockefeller University, New York, New York 10021

Received 14 October 1986/Accepted 23 January 1987

Newly synthesized peroxisomal proteins enter preexisting peroxisomes posttranslationally in vivo, generally without proteolytic processing. An efficient reconstitution of this process in vitro together with cloned DNAs for peroxisomal proteins would make possible investigation of the molecular information that targets proteins to peroxisomes. We have previously reported the isolation of clones for *Candida tropicalis* peroxisomal proteins; here we describe the association (and possible import) of peroxisomal proteins with peroxisomes in vitro. *C. tropicalis* was grown in a medium containing Brij 35, resulting in the induction of a moderate number of medium-sized peroxisomes. These peroxisomes, isolated in a sucrose gradient, had a catalase latency of 54% and were sufficiently stable to be concentrated and used in an import assay. The reticulocyte lysate translation products of total RNA from oleate-grown cells were incubated with the peroxisomes at 26°C in the presence of 50 mM KCl, protease inhibitors, 0.5 M sucrose, 2.5 mM MOPS (morpholinepropanesulfonic acid) (pH 7.2), and 0.5 mM EDTA. Ten major translation products (which could be immunoprecipitated with antiserum against peroxisomal protein) became progressively associated with the peroxisomes during the first 30 min of incubation (some up to ~70%). These include acyl coenzyme A oxidase and the trifunctional protein hydratase-dehydrogenase-epimerase. This association did not occur at 4°C nor did it occur if the peroxisomes were replaced with mitochondria.

All peroxisome proteins studied to date are synthesized on free polysomes and are imported into the organelle postranslationally. New peroxisomes form by division from preexisting peroxisomes (22). This is analogous to the synthesis of chloroplast and mitochondrial proteins, which are similarly encoded in the nucleus. Recent work on mitochondrial and chloroplast biogenesis indicates that these proteins contain an amino-terminal topogenic transit peptide which is involved in targeting the protein to the organelle and is generally proteolytically cleaved upon translocation of the polypeptide into the organelle (5, 8, 16, 19, 27, 28).

Peroxisomal biogenesis differs from that of mitochondria and chloroplasts in that most peroxisomal proteins are synthesized at their final sizes (22). There are some exceptions that are made as larger precursors, but in at least two of these cases, processing to the mature form is not linked to the import process (4, 22). No information is yet available on possible structural sequences which may be important in directing proteins to peroxisomes.

Schatz and colleagues (17, 18), among other, have demonstrated that with an efficient in vitro import system and the manipulation of cloned genes encoding mitochondrial proteins it is possible to investigate the targeting of proteins to an organelle. These powerful tools have not yet been exploited for peroxisome biogenesis. In large part, this is due to the fact that peroxisomes are relatively fragile, making their isolation and subsequent experimental manipulation difficult. Furthermore, peroxisomes represent a much smaller proportion of the normal (noninduced) cell protein than do mitochondria. Nevertheless, limited in vitro posttranslational import of proteins into peroxisomes or glyoxysomes (a specialized type of plant peroxisome) has been accomplished in several cases (10, 14, 21, 31).

Candida tropicalis is an attractive organism with which to pursue an investigation of this sort. Peroxisomes can be strongly induced by growth of this yeast on alkanes (20) or oleic acid (7) or repressed by growth on glucose (7). Oleate-induced peroxisome proliferation is accompanied by a striking induction of ~12 major translatable mRNAs, many of which code for peroxisomal proteins (12). These proteins include catalase and enzymes of the peroxisomal β-oxidation system: acyl coenzyme A (CoA) oxidase (AOx) and the trifunctional enoyl-CoA hydratase–β-hydroxyacyl-CoA dehydrogenase–β-hydroxyacyl-CoA epimerase. Each of these proteins is synthesized at its final size (12).

To investigate the targeting of these proteins to peroxisomes, we first constructed and isolated cDNA clones encoding AOx, catalase, and other peroxisomal proteins (26; R. R. Rachubinski, Y. Fujiki, and P. B. Lazarow, Biochim. Biophys. Acta, in press). Here we report the establishment of yeast growth and fractionation conditions which make it possible to isolate stable peroxisomes and use them in an in vitro import assay. We describe the efficient posttranslational association of several cell-free translation products (including AOx and hydratase-dehydrogenase-epimerase) with these peroxisomes.

MATERIALS AND METHODS

Cultivation of *C. tropicalis* and purification of peroxisomes. *C. tropicalis* pK233 (ATCC 20336) was grown at 30°C in the media listed in Table 1. Cells were harvested in the midst of exponential growth which was 18 h for oleate-grown cells and 9 to 10 h for those grown in other media.

Conversion of cells to spheroplasts, homogenization, and preparation of a crude mitochondrial fraction by differential centrifugation were as described previously (12). For electron microscopy and antibody preparation, peroxisomes were further purified by isopycnic centrifugation in a discontinuous Nycodenz gradient (2 ml of 17%, 3 ml of 28%, 6 ml of 35%, 3 ml of 50% (wt/vol) Nycodenz in 5 mM Tris hydrochloride [pH 7.5]–3 mM KCl–0.3 mM EDTA–0.1%

* Corresponding author.

TABLE 1. Induction of catalase in *C. tropicalis*

Medium	Cell yield (g/liter of culture)[a]	Catalase activity of (U/g of cells)
3% Yeast extract	4.34 ± 0.87 (3)	2.19 ± 1.08 (4)
YP medium[b]	3.10 (1)	1.49 (1)
YP medium + 1% Brij 35	4.70 ± 0.92 (8)	5.13 ± 1.07 (5)
YP medium + 1% Brij 35 + 1% oleic acid	8.67 ± 1.41 (3)	15.57 ± 0.59 (3)
YP medium + 1% Brij 35 + 2% glucose	13.9 (1)	0.13 (1)

[a] Number of experiments is in parentheses.
[b] YP medium is 0.3% yeast extract, 0.5% Bacto-Peptone (Difco Laboratories, Detroit, Mich.), 0.5% K_2HPO_4, 0.5% KH_2PO_4.

ethanol). Centrifugation was for 1 h at 35,000 rpm (98,000 × *g*) in a Sorvall TV-865B vertical rotor.

For in vitro import assays (done in a sucrose medium), peroxisomes were further purified in a linear sucrose gradient (density span, 1.15 to 1.25 g/ml) in a Beaufay zonal rotor (23). The gradient solutions contained 2.5 mM MOPS (morpholinepropanesulfonic acid) (pH 7.2), 0.5 mM EDTA, and 0.1% ethanol. Fractions containing high catalase activity were combined (~4.5 ml) and diluted with 4 volumes of 0.5 M sucrose containing 2.5 mM MOPS (pH 7.2) and 0.5 mM EDTA (import buffer). The peroxisomes were then pelleted by centrifugation (15 min at 13,000 rpm [20,000 × *g*] in a Sorvall SS-34 fixed-angle rotor) and resuspended in ~0.5 ml of import buffer. Peak mitochondrial fractions were similarly diluted, concentrated, and used in control experiments.

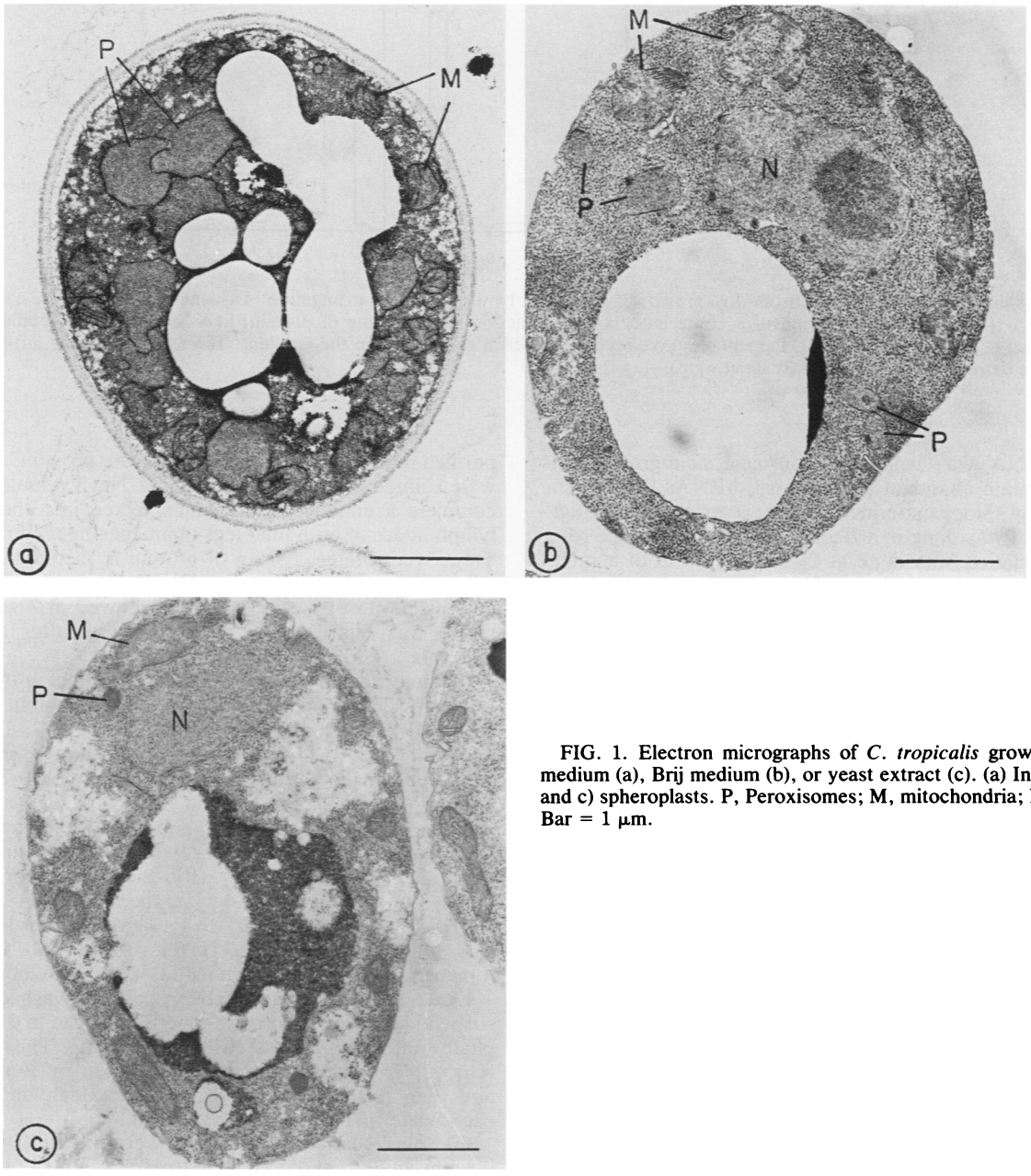

FIG. 1. Electron micrographs of *C. tropicalis* grown in oleate medium (a), Brij medium (b), or yeast extract (c). (a) Intact cell; (b and c) spheroplasts. P, Peroxisomes; M, mitochondria; N, nucleus. Bar = 1 μm.

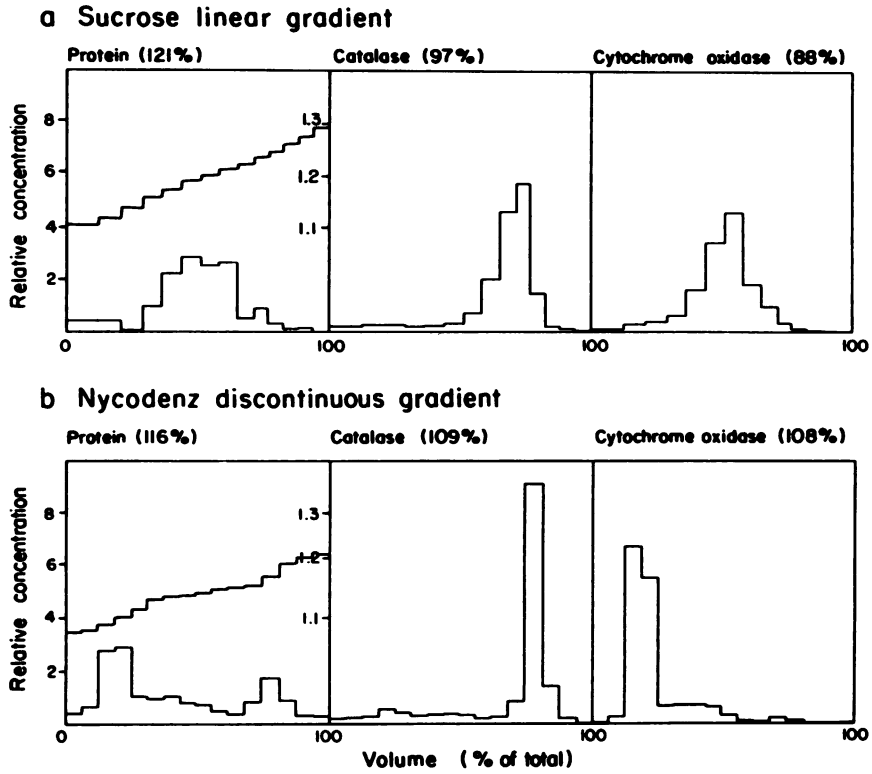

FIG. 2. Isolation of peroxisomes from Brij-grown *C. tropicalis* by isopycnic centrifugation. Distribution of protein, catalase, and cytochrome oxidase after fractionation of a crude mitochondrial fraction (65 to 75 mg of protein) in a linear sucrose gradient (a) or a discontinuous Nycodenz gradient (b). Percentages give recovery of each constituent in the gradient. The staircase in the left-hand boxes indicates the density of each fraction (scale at right).

Total RNA was isolated from oleic acid-grown cells (which contain abundant peroxisomal mRNAs [12]) by the guanidinium thiocyanate method (6) except that centrifugation in CsCl was done in a Beckman 70 Ti fixed-angle rotor (30). Translation was done at 29°C for 90 min in a rabbit reticulocyte lysate protein-synthesizing system treated with micrococcal nuclease (25). Translation had ceased after 90 min.

Posttranslational import assay. After in vitro translation, 2.0 M sucrose was added to the translation mixture to give a final concentration of 0.5 M sucrose. A typical import assay contained 100 μl of the translation mixture, 100 μl of concentrated peroxisomes (0.2 to 0.4 mg of protein), and a final concentration of 0.7 mM of the following protease inhibitors (prepared in dimethyl sulfoxide): leupeptin, chymostatin, antipain, and pepstatin. The assay mixture was divided into four 50-μl aliquots, incubated at 26°C for 0, 15, 30, or 45 min, and then diluted fivefold with import buffer and centrifuged (microcentrifuge, 16,000 × *g*) for 10 min to separate peroxisomes from supernatants. The pellets were solubilized with 1% sodium deoxycholate and 1% Triton X-100 in 0.1 mM phenylmethylsulfonyl fluoride and import buffer. In some experiments, supernatants and pellets were analyzed directly by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), fluorography, and densitometric scanning. In other experiments, the fractions were first subjected to immunoprecipitation. In preliminary experiments, ribosomes were removed by ultracentrifugation before the import assay; this had no effect.

Preparation of antiserum and immunoprecipitation. Highly purified peroxisomes (50 μg) from oleate-grown *C. tropicalis* were diluted into 100 μl of 0.15 M NaCl, emulsified with complete Freund adjuvant, and injected into the popliteal lymph nodes of both hind legs of an anesthetized rabbit (15). Three weeks later, 100 μg of protein in incomplete Freund adjuvant was injected subcutaneously. A further subcutaneous injection of 20 μg of protein followed in 2 weeks. The rabbit was bled 1 week later and thereafter at 2-week intervals.

Immunoprecipitations were done overnight at 0°C with 75 μl of antiserum and 35 μg of peroxisome protein in 0.15 M NaCl–10 mM sodium phosphate buffer (pH 7.2)–1% sodium deoxycholate–1%Triton X-100. The immunoprecipitates were centrifuged through layers of 0.5 and 1.0 M sucrose (29).

Other methods. Catalase (2) and cytochrome oxidase (23) were assayed as described. Protein was determined by the Bio-Rad dye-binding assay (Bio-Rad Laboratories, Richmond, Calif.) with bovine IgG as the standard.

SDS-PAGE was done in 7 to 15% gradient acrylamide slab gels (9). Immunoblotting (11), fluorography (3), and densitometric scanning (11) were as described previously.

Electron microscopy. (i) Cells. A pellet of washed cells was suspended in 0.75% potassium permanganate in 0.1 M phosphate buffer and incubated for 1 h at 4°C. The cells were removed from the permanganate solution by centrifugation and then further fixed in 2.5% glutaraldehyde in 0.1 M cacodylate (pH 7.4) at 4°C overnight.

(ii) Spheroplasts. Washed cells were fixed in 5% glutaraldehyde in 0.1 M cacodylate (pH 7.4) for 1 h at room

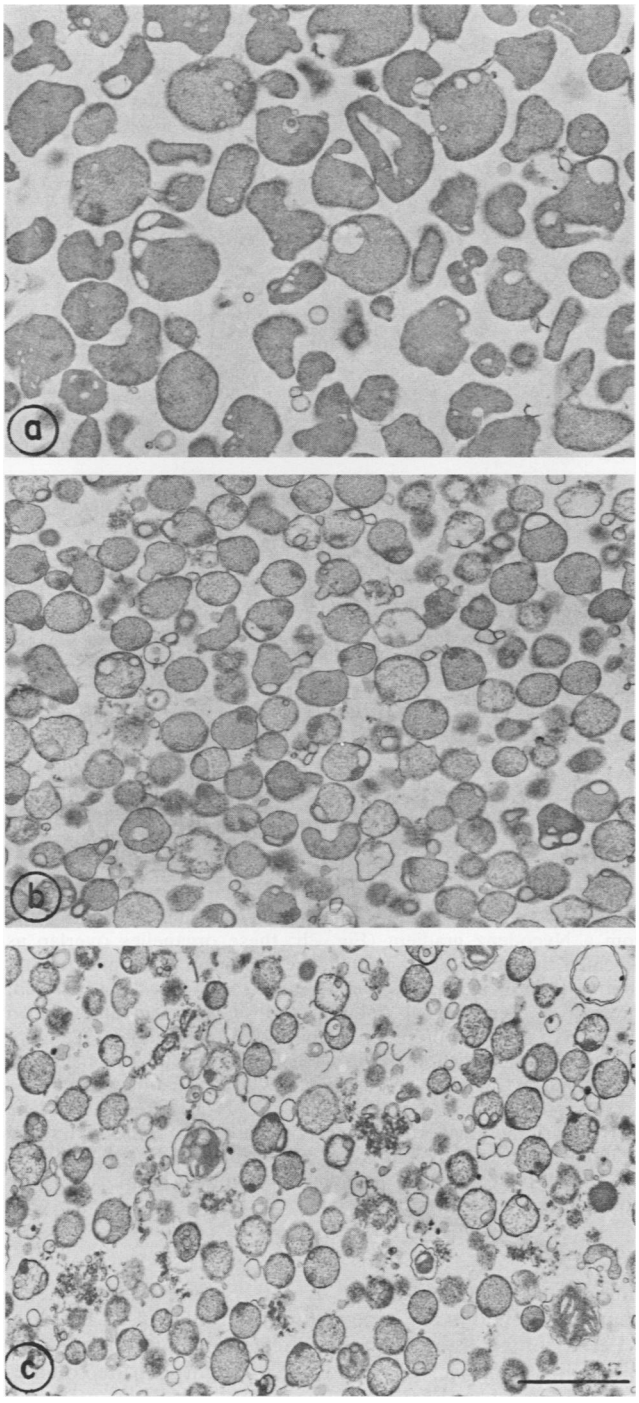

FIG. 3. Electron micrographs of purified peroxisomes from *C. tropicalis* grown on oleic acid (a), Brij (b), yeast extract (c). Peroxisomes were isolated in Nycodenz. Bar = 1 μm.

temperature. Fixed cells were subjected to treatment with 0.2 mg of Zymolyase-100T per ml for 30 to 60 min at 37°C to remove the cell wall. Spheroplasts were centrifuged and suspended in 2.5% glutaraldehyde in 0.1 M cacodylate buffer and left at 4°C overnight.

(iii) Fractions. Fractions were prepared exactly as described previously (1).

All samples were postfixed in 1% osmium tetroxide, stained with uranyl acetate, dehydrated, and embedded in Epon 812.

RESULTS

Choice of growth medium. In preliminary experiments, we found that the abundant large peroxisomes from oleate-grown *C. tropicalis* (12) were too fragile for routine use in cell-free protein import experiments. Therefore, a variety of growth media were surveyed to find conditions in which peroxisomes could be induced substantially but with a smaller mean diameter to improve their stability. Induction was first assessed by assaying catalase, a characteristic peroxisomal marker enzyme. A growth medium containing 1% Brij 35 (Table 1) gave a reasonable yield of cells and induced catalase to one-third the activity obtainable with oleate medium (which also contains 1% Brij 35) (Table 1). Electron microscopy (Fig. 1b) demonstrated the presence in Brij-grown cells of a moderate number of peroxisomes with a size distinctly smaller than that of the more abundant peroxisomes in oleate-grown cells (Fig. 1a). *C. tropicalis* grown on 3% yeast extract also contained moderate numbers of peroxisomes (Fig. 1c), but the catalase activity was only ~40% of that in Brij-grown cells (Table 1).

Purification and stability of peroxisomes. Peroxisomes were purified by differential centrifugation followed by equilibrium density centrifugation in gradients of sucrose or Nycodenz. A good separation of peroxisomes (catalase as marker enzyme) and mitochondria (cytochrome oxidase as marker enzyme) was obtained in a linear sucrose gradient (Fig. 2a). An even better separation occurred in a discontinuous Nycodenz gradient (Fig. 2b). The density of *C. tropicalis* peroxisomes in Nycodenz (1.19 g/ml) was less than in sucrose (1.23 g/ml).

Electron microscopically, peroxisomes purified in Nycodenz from Brij-grown *C. tropicalis* were highly pure and homogeneous in appearance (Fig. 3b). Their diameters were approximately 0.3 to 0.4 μm, distinctly smaller than the 0.7 to 0.9 μm of oleate-induced peroxisomes (Fig. 3a). Peroxisomes purified from cells grown on yeast extract were also ~0.3 μm in diameter but had reduced matrix content (Fig. 3c).

The polypeptide compositions of peroxisomes induced by Brij 35 and by oleate were similar (data not shown). The Brij-induced peroxisomes contained large amounts of the proteins catalyzing β-oxidation, though less than after oleate induction. This suggests that the lauric acid residues in the Brij 35 were used as a carbon source. The Brij 35 also caused a threefold increase in catalase activity above that found in cells grown in YP medium alone (Table 1).

For Nycodenz-purified peroxisomes, the relative specific activity of catalase (specific activity in peroxisomes/specific activity in homogenate) was 12.3. Since the peroxisomes are essentially pure by electron microscopy (Fig. 3b) this implies that in Brij-grown *C. tropicalis*, peroxisomes represent 100%/12.3 = 8.1% of the yeast spheroplast protein. The relative specific activity of catalase in peroxisomes purified in sucrose was 12.0. The peak peroxisomal fraction contained 1% of the cytochrome oxidase present in the gradient in the case of Nycodenz and 2.3% in the case of sucrose. The polypeptide patterns of the peroxisomes isolated in the two media were very similar (data not shown). These data indicate that the peroxisomes isolated in sucrose are essentially as pure as those isolated in Nycodenz.

The stability of the purified peroxisomes was determined by measuring the latency of catalase (that is, the percentage

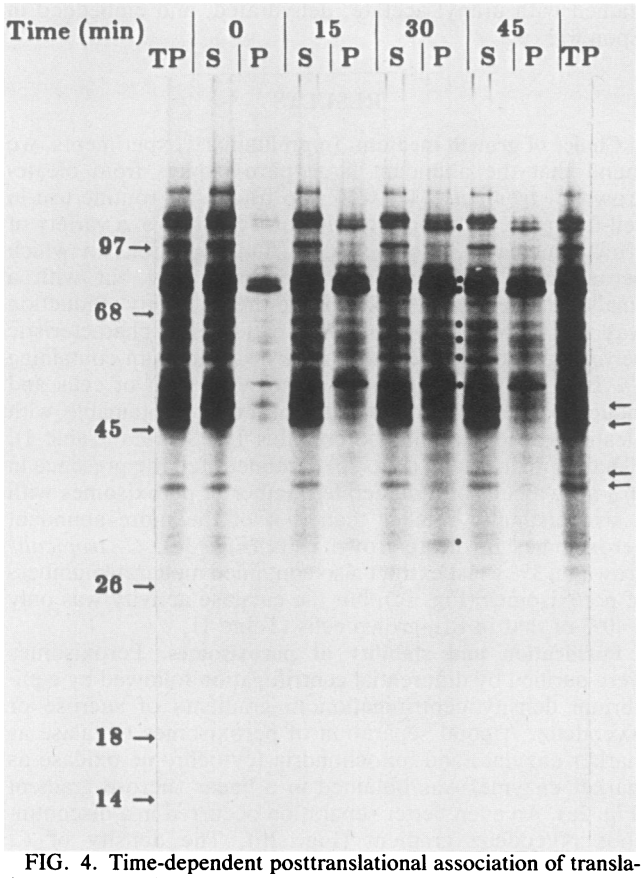

FIG. 4. Time-dependent posttranslational association of translation products with peroxisomes. Translation products (25 μl) were incubated with peroxisomes (~35 μg of protein) at 26°C for the times indicated, and then the peroxisomes (P) were separated from the supernatants (S) by centrifugation. Some 20% of the pellet and 5% of the supernatant was analyzed by SDS-PAGE and fluorography. TP, Total translation products (1 μl). Dots indicate translation products associated with the peroxisome fraction; arrows indicate products remaining in the supernatant. Arrows at the left indicate the positions of the molecular mass markers (×10³).

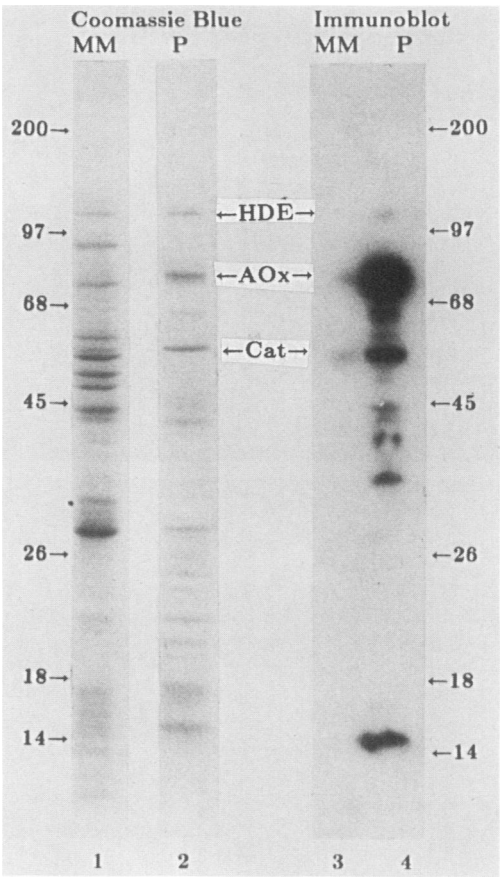

FIG. 5. Specificity of the antiserum against *C. tropicalis* peroxisomal proteins. MM, Mitochondrial-plus-microsomal fraction from a Nycodenz gradient (Fig. 2b; these organelles overlap as indicated by the cytochrome oxidase [mitochondrial marker] and esterase [microsomal marker] distributions in the gradient [data not shown]). P, Peroxisomal fraction. Left, Coomassie blue-stained SDS-PAGE (75 μg of protein per lane). Right, Immunoblot (25 μg of protein was electrophoresed in each lane). The microsomal-plus-mitochondrial fraction also contained ~9% peroxisome protein. As a result, peroxisomal AOx and catalase (Cat) are detectable in lane 3. HDE, Trifunctional protein catalyzing three reactions in fatty acid catabolism: enoyl-CoA hydratase, β-hydroxyacyl-CoA dehydrogenase, and β-hydroxyacyl-CoA epimerase. Numbers with arrows are molecular mass markers (×10³).

of the total catalase activity that is not assayable when the peroxisome membrane is intact and which appears when the membrane is disrupted by detergent). Peroxisomes were first prepared by isopycnic centrifugation through a discontinuous Nycodenz gradient (see Materials and Methods). Catalase latency was low (less than 20%) irrespective of the growth medium used for induction. When 5% Ficoll and 0.6 M sorbitol were included in the Nycodenz gradient, the medium-sized peroxisomes from cells grown in Brij medium were more stable (38% latency), but the larger oleate-induced peroxisomes were still fragile (14% latency). The most stable peroxisomes (54% catalase latency) were those isolated from Brij-grown cells fractionated in a sucrose-MOPS-EDTA gradient in a Beaufay zonal rotor (Fig. 2a). This method was adopted for preparing peroxisomes for import studies, and to maintain peroxisome integrity, the import assay was carried out in a sucrose medium.

Posttranslational association of peroxisomal polypeptides with peroxisomes. Total yeast RNA was translated in vitro with [^{35}S]methionine as the label; thereafter the translation mixture was incubated for 0 to 45 min at 26°C with concentrated purified peroxisomes. Many ^{35}S-labeled polypeptides became associated with the sedimentable organelles in a time-dependent fashion (Fig. 4, dots), while other proteins clearly remained in the supernatant fraction (Fig. 4, arrows).

Peroxisomal translation products were immunoprecipitated with an antiserum raised against highly purified oleate-induced peroxisomes (isolated as in Fig. 2b). This antiserum recognized many of the peroxisomal proteins in an immunoblot analysis (Fig. 5, compare lanes 2 and 4) but did not react with mitochondrial or microsomal proteins (Fig. 5, lanes 1 and 3).

The antiserum immunoprecipitated 10 ^{35}S-labeled translation products that are associated with peroxisomes after 30 min of incubation at 26°C (Fig. 6). These were not found in the peroxisome pellet in the zero-incubation control, but were present in smaller amounts at 15 min. Extending the incubation to 45 min did not increase the association; on the contrary, a slight decrease was reproducibly observed.

At least seven of these immunoprecipitated translation products corresponded to translation products identified by

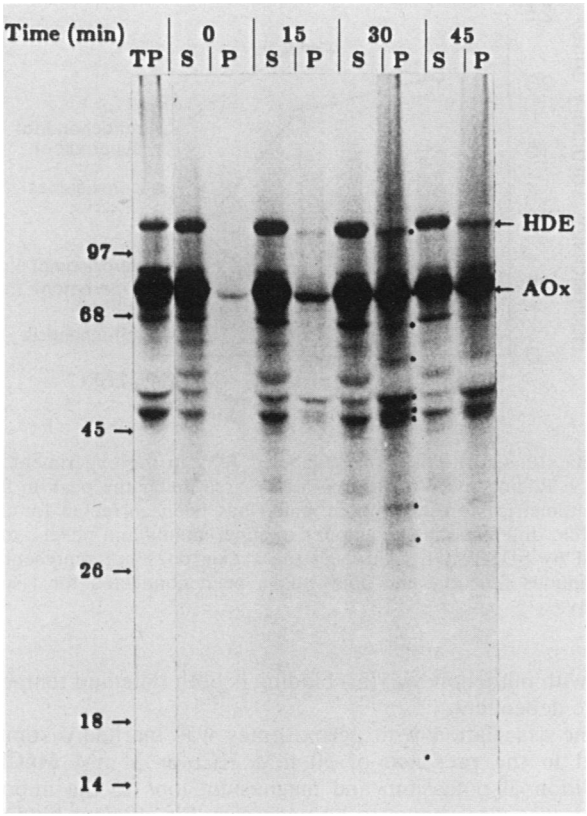

FIG. 6. Immunoprecipitation of translation products. The import assay was the same as for Fig. 4. Peroxisomal translation products were immunoprecipitated from equivalent amounts of supernatants (S) and pellets (P) (12.5% of each) and analyzed by SDS-PAGE and fluorography. TP, Immunoprecipitation of an equivalent amount of translation products; HDE, as defined in the legend to Fig. 5. Numbers with arrows are molecular mass markers ($\times 10^3$).

Fujiki et al. (12) that comigrate in SDS-PAGE with peroxisomal polypeptides of oleate-induced *C. tropicalis*. The radioactivity in the most prominent band among them, AOx, was quantitated by scanning densitometry of the fluorogram

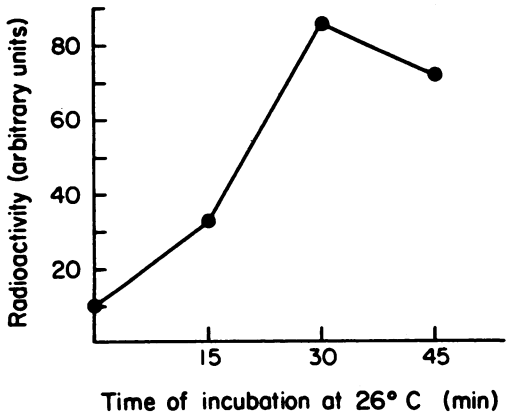

FIG. 7. Kinetics of association of AOx with peroxisomes. ^{35}S-labeled AOx was quantitated by scanning densitometry of the fluorogram of the immunoprecipitated proteins (Fig. 6). Radioactivity is expressed as the area of each peak in arbitrary units.

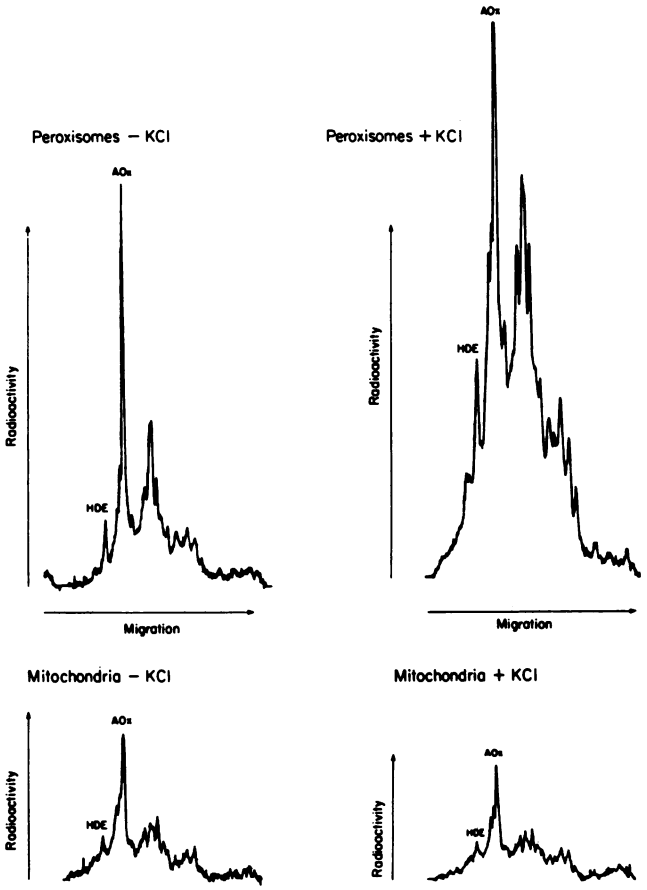

FIG. 8. Effect of inclusion of 50 mM KCl in the assay; organelle specificity. Incubations were done as described in the legend to Fig. 4 at 26°C for 30 min, in the presence (right) or absence (left) of 50 mM KCl. Top, Peroxisomes (60 μg); bottom, mitochondria (53 μg). Samples were analyzed by SDS-PAGE, fluorography, and scanning densitometry. HDE, Defined in the legend to Fig. 5.

(Fig. 7). The association appeared to be approximately linear for the first 30 min, and then a small decrease was observed.

Organelle specificity and effect of KCl. KCl (50 mM) strikingly increased the extent of association of several translation products (including hydratase-dehydrogenase-epimerase and AOx) with peroxisomes (Fig. 8, top). In control experiments there was little association of the peroxisomal translation products with mitochondria (Fig. 8, bottom left). This association was reduced by KCl (Fig. 8, bottom right).

Temperature dependence. No appreciable association of translation products with peroxisomes occurred if the incubation was done at 4°C (Fig. 9, left). In this experiment, which included 50 mM KCl, a clear decrease in several of the ^{35}S-labeled translation products remaining in the supernatant after a 26°C incubation was noted, concomitant with the appearance of these products in the peroxisomal pellet (Fig. 9, dots). These bands, although not immunoprecipitated in this experiment, are clearly peroxisomal proteins corresponding in size to 6 of the 10 immunoprecipitated proteins in Fig. 6. The AOx data were quantitated by densitometric scanning, revealing a strong, temperature-dependent shift of ~70% of the ^{35}S-labeled product from supernatant to pellet (Fig. 10). A similar shift is clear from the densitometric scans (data not shown) for the other peroxisomal polypeptides

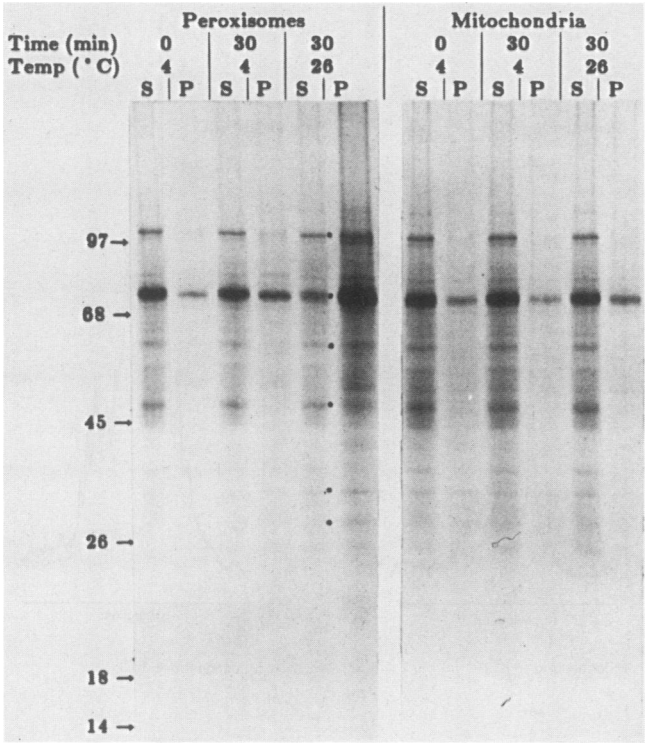

FIG. 9. Temperature dependence and organelle specificity. Incubations were done with peroxisomes (60 μg of protein) or mitochondria (64 μg of protein) in the presence of 50 mM KCl. Peroxisomes (P) and supernatants (S) were analyzed as described in the legend to Fig. 4. Dots indicate polypeptides that decreased in the supernatant and appeared in the pellet (confirmed by densitometric scanning). Numbers with arrows show molecular mass markers ($\times 10^3$).

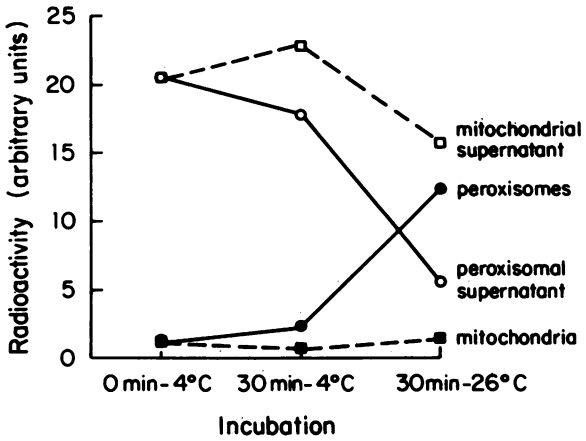

FIG. 10. Quantitation of ^{35}S-labeled AOx in the experiment of Fig. 9. Radioactivity (measured as the area under the peak in the densitometric scan in arbitrary units) has been corrected for the fourfold difference in the amount of supernatants and pellets analyzed by SDS-PAGE. Although the *x* axis does not represent a continuous function, the points have been connected for visual clarity.

indicated in Fig. 9. Nonspecific association with mitochondria was minimal and not temperature dependent (Fig. 9, right; Fig. 10).

Less association of translation products with peroxisomes was observed when the temperature of incubation was raised to 37°C (data not shown). This was probably due to disruption of the peroxisomes or to proteolysis or to both because the Coomassie blue-stained pattern of polypeptides in peroxisomes sedimented at the end of the incubation was abnormal.

DISCUSSION

Optimizing growth and cell fractionation conditions for *C. tropicalis* enabled us to isolate peroxisomes that were stable enough for subsequent in vitro import experiments. We demonstrated a specific binding of ~10 cell-free translation products to peroxisomes and confirmed that these polypeptides are peroxisomal proteins by immunoprecipitation with an antiserum specific for peroxisomal polypeptides. These translation products were sufficiently abundant (owing to induction of their mRNAs by growth of the yeast on oleate) that they could be easily visualized by SDS-PAGE and fluorography, without the need for immunoprecipitation in routine experiments.

Specificity of the binding was established in three ways. (i) Nonperoxisomal translation products are not associated with the peroxisome fractions in these experiments (Fig. 4, arrows); (ii) peroxisomal translation products do not associate with mitochondria; (iii) binding is both time and temperature dependent.

The association with peroxisomes was markedly stimulated in the presence of 50 mM KCl or 3 mM $MgCl_2$. Addition of potassium and magnesium ions to the import assay was also shown to be necessary for efficient binding and transport of the ornithine carbamoyltransferase precursor into rat liver mitochondria (24). Our experiments showed that 50 mM KCl reduces nonspecific binding of translation products coding for peroxisomal proteins to mitochondria (Fig. 8). This agrees with the results of Gasser et al. (13) who showed that reisolation of mitoplasts in the presence of 50 mM KCl eliminated nonspecific adsorption of proteins to their membrane.

This import assay contained no energy supplements to what is supplied by the reticulocyte lysate mixture (which includes GTP, ATP, and an ATP-regenerating system). We know that the ATP concentration at the end of the 90 min of cell-free protein synthesis is 1.2 mM (T. Imanaka, unpublished data). Whether it participates in this association and possible import process is not yet established.

Protease resistance (that can be abolished by detergent) is the criterion generally accepted for establishing that the posttranslational association of a translation product with an organelle is true import rather than binding to the membrane. In the present experiments, four protease inhibitors were included in the import assay to inhibit the active endogenous proteases of *C. tropicalis*. Zymolyase-100T, the impure enzyme preparation used to digest the yeast cell wall, also contains proteases according to the supplier (The Research Laboratories, Kirin Brewery Co., Ltd., Tokyo, Japan). The presence of these inhibitors precludes, for the moment, evaluating the protease resistance of the translation products associated with the peroxisomes. The fact that KCl stimulates this association makes it likely that it is true import, because one might expect binding to be reduced by KCl. This problem is being investigated further in experiments in which individual cloned DNAs are expressed in vitro because it is easier to optimize a proteolysis protocol for one protein, rather than attempting to digest the total translation products (10).

Demonstration of import of proteins into microbodies in vitro has proved difficult, and in the few examples reported thus far the import has been inefficient (e.g., ~20% for malate dehydrogenase into watermelon glyoxysomes) (14). For the first time we demonstrated efficient (up to 70%) in vitro association (import?) of many peroxisomal proteins to peroxisomes of *C. tropicalis*. Prominent among these is the major cell-free translation product, a 76,000-dalton polypeptide which corresponds to AOx (12). A cDNA clone for AOx (26) has been modified and expressed in vitro (G. M. Small, T. Imanaka, and P. B. Lazarow, J. Cell Biol. **103**:524a, 1986). The products are being used in the cell-free import assay to assess possible targeting information contained within the protein sequence.

ACKNOWLEDGMENTS

We thank Gary Richo for careful technical assistance.

This research was supported by National Science Foundation grant DCB83-09222. P.B.L. was the recipient of an Established Fellowship from the New York Heart Association.

LITERATURE CITED

1. **Alexson, S. E. H., Y. Fujiki, H. Shio, and P. B. Lazarow.** 1985. Partial disassembly of peroxisomes. J. Cell Biol. **101**:294–304.
2. **Baudhuin, P., H. Beaufay, Y. Rahman-Li, O. Z. Sellinger, R. Wattiaux, P. Jacques, and C. de Duve.** 1964. Tissue fractionation studies. 17. Intracellular distribution of monoamine oxidase, aspartate aminotransferase, alanine aminotransferase, D-amino acid oxidase and catalase in rat-liver tissue. Biochem. J. **92**:179–184.
3. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
4. **Borst, P.** 1986. How proteins get into microbodies (peroxisomes, glyoxysomes, glycosomes). Biochim. Biophys. Acta **866**:179–203.
5. **Cashmore, A., L. Szabo, M. Timko, A. Kausch, G. Van den Broeck, P. Schreier, H. Bohnert, L. Herrera-Estrella, M. Van Montagu, and J. Schell.** 1985. Import of polypeptides into chloroplasts. Bio/Technology **3**:803–807.
6. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
7. **Dommes, P., V. Dommes, and W.-H. Kunau.** 1983. β-Oxidation in *Candida tropicalis*. Partial purification and biological function of an inducible 2,4-dienoyl coenzyme A reductase. J. Biol. Chem. **258**:10846–10852.
8. **Ellis, R. J.** 1981. Chloroplast proteins: synthesis, transport, and assembly. Annu. Rev. Plant Physiol. **32**:111–137.
9. **Fujiki, Y., A. L. Hubbard, S. Fowler, and P. B. Lazarow.** 1982. Isolation of intracellular membranes by means of sodium carbonate treatment. Application to endoplasmic reticulum. J. Cell Biol. **93**:97–102.
10. **Fujiki, Y., and P. B. Lazarow.** 1985. Post-translational import of fatty acyl-CoA oxidase and catalase into peroxisomes of rat liver *in vitro*. J. Biol. Chem. **260**:5603–5609.
11. **Fujiki, Y., R. A. Rachubinski, and P. B. Lazarow.** 1984. Synthesis of a major integral membrane polypeptide of rat liver peroxisomes on free polysomes. Proc. Natl. Acad. Sci. USA **81**:7127–7131.
12. **Fujiki, Y., R. A. Rachubinski, A. Zentella-Dehesa, and P. B. Lazarow.** 1986. Induction, identification, and cell-free translation of mRNAs coding for peroxisomal proteins in *Candida tropicalis*. J. Biol. Chem. **261**:15787–15793.
13. **Gasser, S. M., G. Daum, and G. Schatz.** 1982. Import of proteins into mitochondria. Energy-dependent uptake of precursors by isolated mitochondria. J. Biol. Chem. **257**:13034–13041.
14. **Gietl, C., and B. Hock.** 1984. Import of in-vitro-synthesized glyoxysomal malate dehydrogenase into isolated watermelon glyoxysomes. Planta **162**:261–267.
15. **Goudie, R. B., C. H. W. Horne, and P. C. Wilkinson.** 1966. A simple method for producing antibody specific to a single selected diffusible antigen. Lancet **ii**:1224–1226.
16. **Hay, R., P. Bohni, and S. Gasser.** 1984. How mitochondria import proteins. Biochim. Biophys. Acta **779**:65–87.
17. **Hurt, E. C., B. Pesold-Hurt, and G. Schatz.** 1984. The amino-terminal region of an imported mitochondrial precursor polypeptide can direct cytoplasmic dihydrofolate reductase into the mitochondrial matrix. EMBO J. **3**:3149–3156.
18. **Hurt, E. C., B. Pesold-Hurt, K. Suda, W. Oppliger, and G. Schatz.** 1985. The first twelve amino acids (less than half of the pre-sequence) of an imported mitochondrial protein can direct mouse cytosolic dihydrofolate reductase into the yeast mitochondrial matrix. EMBO J. **4**:2061–2068.
19. **Hurt, E. C., and A. P. G. M. van Loon.** 1986. How proteins find mitochondria and intramitochondrial compartments. Trends Biochem. Sci. **11**:204–207.
20. **Kawamoto, S., C. Nozaki, A. Tanaka, and S. Fukui.** 1978. Fatty acid β-oxidation system in microbodies of *n*-alkane-grown *Candida tropicalis*. Eur. J. Biochem. **83**:609–613.
21. **Kruse, C., J. Frevert, and H. Kindl.** 1981. Selective uptake by glyoxysomes of in vitro translated malate synthase. FEBS Lett. **129**:36–38.
22. **Lazarow, P. B., and Y. Fujiki.** 1985. Biogenesis of peroxisomes. Annu. Rev. Cell Biol. **1**:489–530.
23. **Leighton, F., B. Poole, H. Beaufay, P. Baudhuin, J. W. Coffey, S. Fowler, and C. de Duve.** 1968. The large scale separation of peroxisomes, mitochondria and lysosomes from the livers of rats injected with Triton WR-1339. J. Cell Biol. **37**:482–513.
24. **Miura, S., M. Mori, and M. Tatibana.** 1983. Transport of ornithine carbamoyltransferase precursor into mitochondria. Stimulation by potassium ion, magnesium ion, and a reticulocyte cytosolic protein(s). J. Biol. Chem. **258**:6671–6674.
25. **Pelham, H. R. B., and R. J. Jackson.** 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. **67**:247–256.
26. **Rachubinski, R. A., Y. Fujiki, and P. B. Lazarow.** 1985. Cloning of cDNA coding for peroxisomal acyl-CoA oxidase from the yeast *Candida tropicalis* pK 233. Proc. Natl. Acad. Sci. USA **82**:3973–3977.
27. **Schatz, G., and R. A. Butow.** 1983. How are proteins imported into mitochondria? Cell **32**:316–318.
28. **Schmidt, G. W., and M. L. Mishkind.** 1986. The transport of proteins into chloroplasts. Annu. Rev. Biochem. **55**:879–912.
29. **Taylor, J. M., and R. T. Schimke.** 1973. Synthesis of rat liver albumin in a rabbit reticulocyte cell-free protein-synthesizing system. J. Biol. Chem. **248**:7661–7668.
30. **Turpen, T. H., and O. M. Griffith.** 1986. Rapid isolation of RNA by a guanidinium thiocyanate/cesium chloride gradient method. BioTechniques **4**:11–15.
31. **Zimmermann, R., and W. Neupert.** 1980. Biogenesis of glyoxysomes, Synthesis and intracellular transfer of isocitrate lyase. Eur. J. Biochem. **112**:225–233.