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Organelle Membranes from Germinating Castor Bean Endosperm

II. ENZYMES, CYTOCHROMES, AND PERMEABILITY OF THE GLYOXYSOME MEMBRANE[1]

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ABSTRACT

Glyoxysome ghosts were isolated from germinating castor bean endosperms using established methods. Electron microscopic examination showed that some matrix material was retained within the glyoxysomal membrane. Two cytochrome reductases and phosphorylcholine glyceride transferase co-sedimented with the alkaline lipase, a known component of the glyoxysome membrane, in sucrose gradient centrifugation of osmotically shocked glyoxysomes. The activities of these enzymes in the glyoxysome membranes were compared to those in the endoplasmic reticulum relative to phospholipid content. On this basis, the phosphorylcholine glyceride transferase was 10-fold more active in the endoplasmic reticulum, whereas the lipase was 50-fold more active in the glyoxysome membrane. The cytochrome reductases were only 2-fold more active in the endoplasmic reticulum, indicating that they are components of the two membranes. Difference spectroscopy of the glyoxysome membrane suspension revealed the presence of a *b*5-type cytochrome similar to that found in the endoplasmic reticulum. Since the glyoxysome membrane is apparently derived from the endoplasmic reticulum, components of the endoplasmic reticulum such as these are likely to be incorporated into the glyoxysome membrane during biogenesis.

Enzyme activities involving the cofactors NADH or CoA were measurable in broken, but not in intact, glyoxysomes. Thus, it appears that cofactors for enzymes within the organelle cannot pass through the membrane.

The glyoxysome, a membranous organelle, was first described in germinating castor bean endosperm by Breidenbach and Beevers in 1967 (5). It has since emerged as an important participant in the conversion of stored triglyceride to sucrose in germinating fatty seeds (1). It is conceptually related to the peroxisomes found in leaves (22) and liver (7) in that it contains catalase and H_2O_2-producing oxidases. The glyoxysome is unique in containing the enzymes of the glyoxylate cycle (1, 5) together with those of β-oxidation (6).

The glyoxysome membrane is thought to be derived from the ER because it has been seen to be connected in electron micrographs of developing stages (22), because it has a similar polypeptide composition (3), and because it has the same lipid components (9). Also, precursors, such as [^{14}C]choline, [^{14}C]acetate, or [^{35}S]-methionine, provided to endosperm tissue of germinating castor bean initially appear as lipid and protein in the ER and subsequently in the glyoxysomes (4, 9, 14). Phospholipid synthetic enzymes and ribosomes are associated with the ER but are absent from the glyoxysome, further evidence that the ER must be responsible for the generation of the glyoxysome membrane lipids and proteins (1, 16, 23).

The metabolic activities of the glyoxysome are enclosed by the membrane which must serve to contain the enzymes and metabolic intermediates. However, the membrane should allow substrates, such as fatty acids, to enter and products, such as succinate, to leave. The lipase in the membrane may accept glycerides from the outside and release free fatty acids inside the organelle. The continued association of several glyoxylate cycle enzymes with glyoxysome "ghosts" following osmotic shock (13) has led to the suggestion that some metabolism takes place on the inner surface of the membrane (2).

Here, we demonstrate that known enzyme components of the ER are also found in the glyoxysome membrane. Also, evidence for the confinement of metabolites and cofactors by the membrane is presented.

MATERIALS AND METHODS

Glyoxysome Membrane Preparation. Glyoxysomes were obtained from castor bean (*Ricinus communis* L.) endosperm after 4 days germination at 30 C in darkness. The glyoxysome fractions were obtained in 51% w/w sucrose after the homogenate (24) from 50 g endosperm had been centrifuged on three linear sucrose gradients [30 ml, 15–60% w/w sucrose, 1 mM EDTA (pH 7.5)] for 2 h at 21,000 rpm in a Beckman SW 25.2 rotor. The combined glyoxysome fractions (7.2 ml) were osmotically shocked by dilution with 2 volumes 0.225 M KCl, 0.05 M Tricine (pH 7.5) and centrifuged again on an identical sucrose gradient or pelleted at 40,000 rpm for 30 min (13).

Analysis of Gradients. Fractions from such gradients were analyzed for sucrose (refractometer), protein (17), phospholipid (10), alkaline lipase (18), phosphorylcholine glyceride transferase (16), NADH (8), and NADPH (16) Cyt *c* reductases.

Cytochrome Spectroscopy. The glyoxysome membrane pellet, resuspended in 0.05 M Tricine (pH 7.5), was analyzed for Cyt content by difference spectroscopy using a Perkin Elmer 555 double-beam spectrophotometer. A crystal of NADH or dithionite was added to the undiluted glyoxysome membrane fraction in the sample cuvette. The absorption spectrum from 400 to 600 nm was scanned with respect to an identical reference sample of the glyoxysome membrane without added reductant.

Latent Activity. Determination of enzyme activities with intact glyoxysomes was performed in reaction mixtures containing 52%

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w/w sucrose, that is, β-hydroxyacyl-CoA-dehydrogenase (19), malate synthetase (12), and malate dehydrogenase (13) activities were measured as previously described but with 52% w/w sucrose in the reaction mixtures. Glyoxysomes were broken by including 0.2% Triton X-100 in the assay or by diluting the glyoxysome sample with 2 volumes 0.225 M KCl. Enzyme activities in the broken glyoxysomes were measured in the presence of the same concentration of sucrose as for the intact organelles.

Electron Microscopy. Glyoxysome ghosts were pelleted at 40,000 rpm for 1 h in a Beckman type 65 rotor, after diluting the original fraction with 2 volumes 0.225 M KCl, 0.05 M Tricine (pH 7.5). The pellet was fixed in Karnovsky's reagent, postfixed in 1% OsO_4, and embedded in Epon-Araldite. The sections were stained with uranyl acetate and lead citrate and examined in a Jeol 100B electron microscope.

RESULTS

Characterization of Glyoxysome Membrane Preparation. When glyoxysomes isolated in 51% w/w sucrose were osmotically shocked in the presence of 0.15 M KCl and centrifuged on a second sucrose gradient, most of the matrix enzymes were solubilized and the glyoxysome membranes were equilibrated at 48% w/w sucrose as originally demonstrated by Huang and Beevers (13). Membranes of liver peroxisomes may also be obtained in this manner (8).

The location of the glyoxysome membranes in the second gradient (Fig. 1) was indicated by the presence of protein, phospholipid, and the characteristic lipase (18). A significant amount of matrix material was visible within the membrane (Fig. 2), confirming that not all of the protein components in this preparation are associated with the membrane. Nevertheless, a large amount of protein was solubilized by the treatment as represented in the left-hand portion of the protein distribution shown in Figure 1. It has been shown previously that, after osmotic shock in 0.15 M KCl, about 20% of the malate synthetase, 20% of the β-hydroxyacyl-CoA dehydrogenase, and 30% of the total glyoxysome protein remain associated with the membranes (13). Under the same conditions, the lipase and phospholipid were not solubilized (Fig. 1).

ER Enzymes in Glyoxysome Membrane. Phosphorylcholine glyceride transferase was very similar to the lipase in its distribution on the gradient (Fig. 1). The bulk of this activity, as well as that of other enzymes involved in phospholipid synthesis, is associated with the ER in this tissue (1, 16).

Two other activities which typify the ER, NADH, and NADPH Cyt *c* reductases (8, 16) also co-sedimented with the glyoxysome membranes. In each case, some of the activity was solubilized.

Each of the ER enzymes had maximal activity in fraction 11 (Fig. 1), whereas the lipase, phospholipid, and protein were maximal in fraction 12. Similar slight differences in equilibrium densities for matrix enzymes have also been seen (11, 13) and are judged insignificant.

Cytochrome Spectroscopy of Glyoxysome Membrane. In the presence of reductant, the glyoxysome membrane suspension (when compared to oxidized membranes) had absorption maxima at 424 and 552 nm and a minimum at 405 nm (Fig. 3). These were apparent when NADH was used as the reductant but were enhanced by the addition of dithionite. This reduced *versus* oxidized difference spectrum was similar to that obtained from ER which had maxima at 442, 526, and 554 nm (15). The absorption spectra indicate that the glyoxysome membrane contains a *b*5-type Cyt like that found in the ER. The glyoxysome membrane preparation seemed to be free of mitochondria which had prominent absorption at 520, 552, and 630 nm (not shown).

Enzyme Activities Relative to Phospholipid Content. Except for the lipase, the specific activities (based on protein content) of all the enzymes shown in Figure 1 were higher in the ER than in the

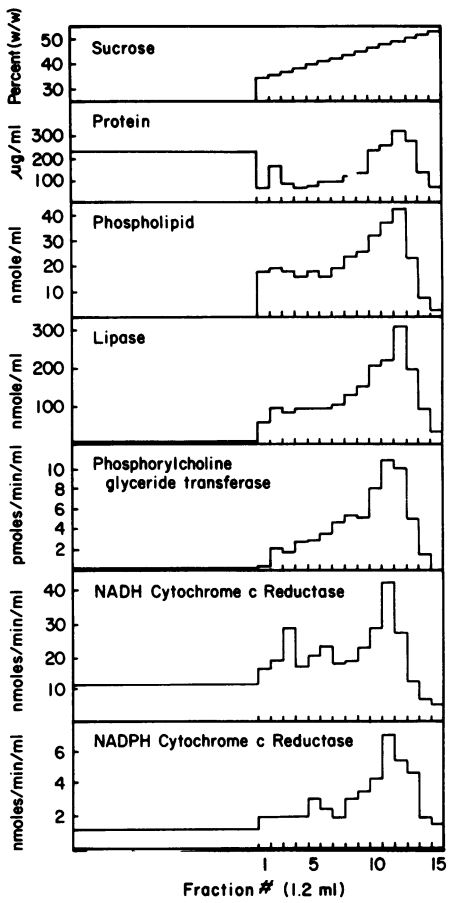

FIG. 1. Glyoxysome membranes in sucrose density gradient centrifugation. Glyoxysomes were separated from other organelles on a prior gradient. The glyoxysome fraction was subjected to osmotic shock in 0.15 M KCl and centrifuged on this gradient. The supernatant, 39 ml, was taken from the top and 1.2-ml fractions were collected.

glyoxysome membranes. The amount of protein relative to phospholipid was greater in the glyoxysome membrane preparation than in a corresponding ER preparation (Table I), probably because some matrix protein remained associated with the glyoxysome membranes (Fig. 2). To exclude nonmembrane protein from consideration, enzyme activities were compared on the basis of phospholipid rather than protein. Phospholipid was chosen because it accounts for 80% of the total acyl lipid in the ER and in the glyoxysome membrane (10). Another 13% of the lipid in each membrane is in the form of free fatty acid, which does not seem to be derived from the phospholipid because the fatty acids are different. The remaining lipids in the two membranes are triglycerides and diglycerides, which also differ from the phospholipid in their fatty acid content (10).

Relative to phospholipid, the lipase was 50-fold more active in the glyoxysome membrane, whereas the phosphorylcholine glyceride transferase was 10-fold more active in the ER (Table I). The Cyt reductases were only about twice as active in the ER. The amounts of Cyt reductases in the glyoxysome membrane cannot be explained by ER contamination. To account for the relative activities observed, one-half of the phospholipid material in the glyoxysome membrane preparation would have to be ER. This is clearly not the case since the relative activities of the lipase and the phosphorylcholine glyceride transferase indicate that at least 90% of the material in the glyoxysome membrane preparation is indeed glyoxysome membrane.

Accessibility of Glyoxysome Enzymes to External Substrates

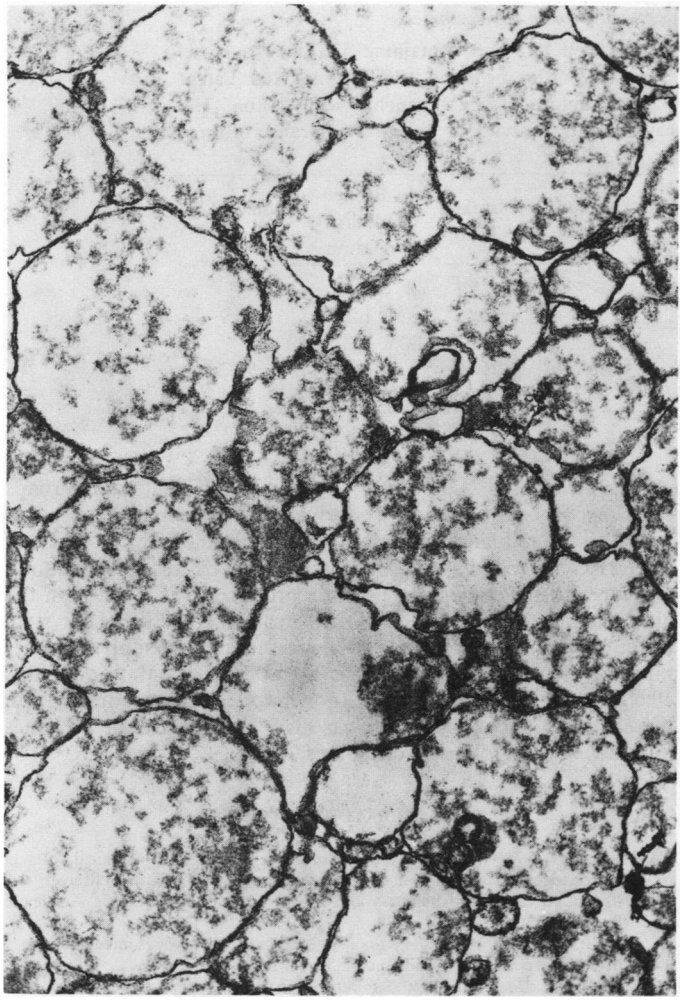

FIG. 2. Thin section of a glyoxysome pellet following osmotic shock in 0.15 M KCl. × 40,000. Bar is 0.2 μm.

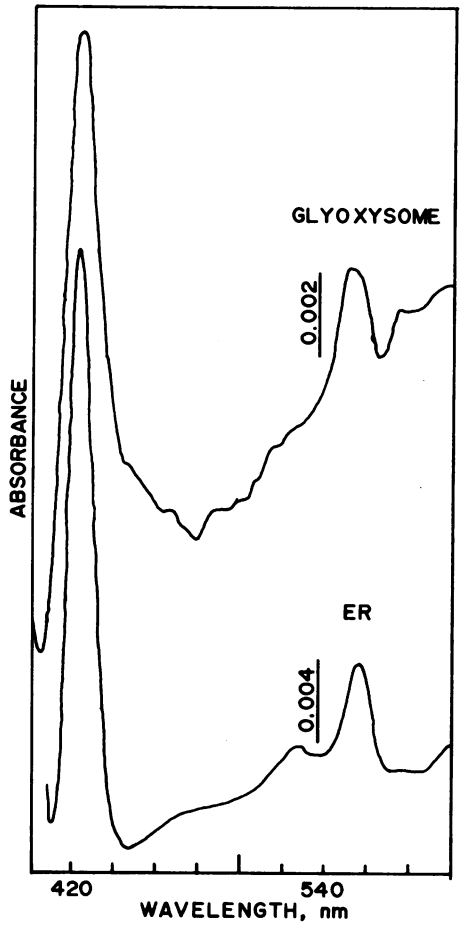

FIG. 3. Reduced *versus* oxidized difference spectroscopy of glyoxysome membranes and ER. Membranes were pelleted from sucrose gradient fractions and resuspended as described under "Materials and Methods." Reductant ($Na_2S_2O_4$) was added to 0.6 ml membrane suspension in the sample cuvette. The absorption spectrum was recorded with reference to an identical unreduced sample. The bars indicate the different *A* scales for the glyoxysome membranes and the ER.

and Cofactors. To determine the access of substrates and cofactors to enzymes contained in the glyoxysome, enzyme activities in 52% sucrose (initial rates) were compared before and after organelle rupture. The enzymes examined are matrix components which become soluble when the organelles are broken (13). The activity of each of the enzymes was greatly increased after rupture by detergent or by dilution (Table II). When glyoxysomes broken by dilution were analyzed for enzyme activity in the presence of detergent, malate dehydrogenase was inhibited 30% and β-hydroxyacyl-CoA dehydrogenase was stimulated 30%. Most of the stimulation by the detergent represents breakage of the organelles rather than a direct enhancement of the reaction. The β-hydroxyacyl-CoA dehydrogenase was also found to be latent in the original homogenate (13% w/w sucrose). The glyoxysome were broken by freezing and thawing the homogenate several times or by including detergent in the reaction mixture. Malate dehydrogenase latency in the homogenate could not be studied in this way because the enzyme is also found in the cytosol and the mitochondria. Malate synthetase activity appeared to be 85% latent in the homogenate, but these measurements were complicated by nonenzymic reactions.

The data suggest that at least one of the participants in each reaction did not have free access to the enzyme in intact glyoxysomes. The reactions tested involved NADH, CoA, glyoxylate, and oxaloacetate.

Table I. *Enzyme Activities Relative to Phospholipid*

The ER fraction from a sucrose gradient was diluted with 0.225 M KCl and recentrifuged on a second sucrose gradient in the same manner as for the glyoxysome membranes.

Enzyme	Glyoxysome Membrane	ER
	Units/nmol phospholipid	
Protein	7.15[a]	1.42[a]
Lipase	7.30[b]	0.15[b]
Phosphorylcholine glyceride transferase	0.24[c]	2.52[c]
NADH Cyt *c* reductase	0.64[b]	1.40[b]
NADPH Cyt *c* reductase	0.13[b]	0.22[b]

[a] Measured in μg.
[b] Measured in nmol/min.
[c] Measured in pmol/min.

DISCUSSION

The glyoxysome membrane is very similar to that of the ER. Both are made up primarily of phosphatidylcholine and phosphatidylethanolamine having identical fatty acid compositions (10). Most of the enzymes of the ER, including Cyt reductases, a *b*5-type Cyt, and some phosphorylcholine glyceride transferase, are

Table II. *Enzyme Activities of Intact and Broken Glyoxysomes*

Glyoxysomes from a sucrose gradient were assayed in reaction mixtures containing 52% w/w sucrose. Glyoxysomes were broken by dilution with 2 volumes 0.225 M KCl or by including 0.2% Triton X-100. Latent activity is that percentage of activity measurable only upon rupture of the organelles with Triton. The concentration of sucrose was maintained at 52% in all measurements. Activities are the average range of three separate initial-rate measurements.

Enzyme	Substrates	Intact	Broken Dilution	Broken Triton	Broken Dilution + Triton	Latent Activity
			mmol/min·ml			%
β-Hydroxyacyl-CoA dehydrogenase	NADH, acetoacetyl-CoA	0.23	0.94	1.52	1.29	84.9
Malate synthetase	CoA, glyoxylate	0.48	nd[a]	26.60	nd[a]	98.2
Malate dehydrogenase	NADH, oxalacetate	0.08	0.51	0.28	0.34	71.4

[a] Not determined.

also found in the glyoxysome membranes. Similarly, liver peroxisome membranes have been shown to contain Cyt reductase (8) and Cyt *b*5 (20).

There are also some important differences between the glyoxysome membrane and the ER. The glyoxysome membrane contains more lipase and less phosphorylcholine glyceride transferase. It does not bind ribosomes (23). Even after osmotic shock and exposure to high salt concentration, the glyoxysome membranes move to a higher density in sucrose gradient centrifugation. This suggests that the glyoxysome membrane is easily penetrated by sucrose, whereas the ER vesicles are not. The residual matrix protein in the glyoxysome membrane preparation will also affect its equilibrium density.

The ER is apparently responsible for the generation of the glyoxysome membrane. The phospholipids found in the glyoxysome membrane are originally synthesized in the ER (9, 16). Glyoxysomal proteins are also produced in the ER, including both membrane and matrix components (4, 11). Therefore, it is not surprising to find ER enzymes in the glyoxysome membrane inasmuch as, during the production of the glyoxysome membrane, components of the ER may be carried over, selectively or nonselectively. Some of these components may have no functional role in the glyoxysome membrane. Some differentiation of the membrane must occur because, during glyoxysome production, the lipase is specifically included and the phosphorylcholine glyceride transferase is largely excluded. The small amount of lipase in the ER and phosphorylcholine glyceride transferase in the glyoxysome membrane may be indicative of *in vivo* cross-contamination during biogenesis or of *in vitro* cross-contamination during fractionation. However, the amounts of NADH and NADPH Cyt *c* reductases in the glyoxysome membrane suggest that the proteins involved are not excluded during biogenesis and cannot represent preparative contamination. The NADH Cyt *c* reductase activity is likely to be the manifestation of a flavoprotein and the *b*5 Cyt (21).

The latency studies suggest that the glyoxysome membrane is relatively impermeable to cofactors or substrates of enzymes contained within the organelle. Another interpretation of the observations is that the access of external substrates to enzymes in the intact organelle is diffusion-limited, especially in a 52% sucrose medium. Latency in a 13% sucrose medium was also observed in the original homogenate for β-hydroxyacyl-CoA dehydrogenase and malate synthetase. Latency has previously been reported for malate synthetase in glyoxysome membrane ghosts (2), even when sucrose was not included in the reaction medium (13). It seems most likely that the cofactors NADH and CoA, which have mol wt around 700, do not penetrate the membrane. The confinement of CoA derivatives within the glyoxysome would prevent their metabolism via mitochondrial pathways. The possible confinement of NADH reiterates the problem of oxidation of the NADH produced within the glyoxysomes by β-oxidation and the glyoxylate cycle (15). NADH Cyt *c* reductase, the *b*5 Cyt, and perhaps other electron carriers could conceivably provide a mechanism for transporting reductant through the membrane. The activity of NADH Cyt *c* reductase as measured would be less than required to deal with the maximum possible production of NADH in the glyoxysome. However, the physiological acceptor for the system is not likely to be Cyt *c*, and other acceptors might support higher rates of electron transport. The membrane orientation of this electron transport system is not known nor can it be deduced from these studies since no attempt was made to measure the activities in intact *versus* broken glyoxysomes.

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