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Role of the Endoplasmic Reticulum in Glyoxysome Formation in Castor Bean Endosperm[1]

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ABSTRACT

Homogenates of the endosperm of castor bean (*Ricinus communis* var. Hale) were prepared at intervals during germination and fractionated on sucrose gradients. Early in germination when glyoxysomes were being produced, a substantial proportion (50%) of the activities of malate synthetase and citrate synthetase was recovered in the membranes of the endoplasmic reticulum (mean density 1.12 grams per cubic centimeter). This proportion declined to less than 10% at 4 days when the glyoxysomes were fully developed.

Gradient fractions challenged by antiglyoxysome-protein antiserum in double immunodiffusion assay revealed strong antigenic response in the endoplasmic reticulum membranes. The results support the view advanced earlier that glyoxysomes are derived directly from the endoplasmic reticulum.

A striking increase in glyoxysomes accompanies the onset of fat breakdown in the endosperm of the germinating castor bean (2). Previous work on the biogenesis of these organelles has shown that the various phospholipid components of their membranes are synthesized exclusively on the membranes of the ER,[3] suggesting that the glyoxysomal membrane may be derived directly by vesiculation from the ER (7). In this paper the intracellular distribution of distinctive enzymes and proteins of glyoxysomes is examined during early growth and for some of these components, too, the ER appears to be the initial site of deposition.

MATERIALS AND METHODS

Seeds of castor bean (*Ricinus communis* var. Hale) were soaked in running tap water for 1 day and germinated in moist vermiculite in darkness at 30 C.

Homogenization. Homogenization of the endosperm was carried out as previously described (10). The grinding medium contained 100 mM Tricine (pH 7.5), 10 mM KCl, 1 mM EDTA and 20% sucrose (w/w).

Fractionation of Cellular Components. Gradients of sucrose solution (26 ml or 38 ml) increasing linearly from 20 to 48% (w/w) were constructed over a 6-ml cushion of 60% sucrose. Sucrose solutions contained 100 mM Tricine, pH 7.5, 10 mM KCl, and 1 mM EDTA. Gradients were centrifuged and fractionated as described previously (10).

Immunochemical Procedures. Rabbits were immunized with glyoxysomal proteins and boosted as described by Huang *et al.* (6). Whole antiserum was used for gel diffusion analysis.

Ouchterlony plates were prepared with 1% agarose (Calbiochem) in phosphate-buffered saline, pH 7.2, with 0.01% merthiolate as preservative. Diffusion was allowed to proceed 18 to 20 hr at ambient temperature.

Enzyme Assays. Catalase (11), malate synthetase (3), citrate synthetase (3), isocitrate lyase (3), and hydroxy acyl CoA dehydrogenase (13) were assayed as described by others on a Gilford 2000 recording spectrophotometer. NADPH Cyt *c* dehydrogenase activity was measured in a volume of 0.25 ml which contained in μmoles: phosphate buffer, pH 6.7, 5; Cyt *c* (oxidized), 0.02; NADPH, 0.1; KCN, 2.5. The reaction was started by the addition of enzyme.

RESULTS

Enzyme Distribution within Gradients. The amount of glyoxysomal protein and of the distinctive enzymes reaches a peak 4 to 5 days after sowing under the conditions employed (2). Figure 1 shows the distribution of protein and some marker enzymes when the whole homogenate prepared from endosperms of 4-day seedlings was centrifuged on the linear sucrose gradient. The major protein peaks at densities 1.19 g/cc and 1.24 g/cc represent, respectively, mitochondria and glyoxysomes, and malate dehydrogenase activity is present in both of these organelles. Malate dehydrogenase from the cytosol is recovered in the uppermost fractions of the gradient. 3-OH acyl CoA dehydrogenase is present only in the glyoxysomes. The peak of NADPH-Cyt *c* reductase activity at density 1.12 g/cc coincides with the discrete protein band (band B in Ref. 9) which was shown to be comprised of membranes of the ER (10).

Separation of organelles from a similar homogenate from endosperm of 2-day-old seedlings is shown in Figure 2. At this stage of development the amount of protein in mitochondria and glyoxysomes is only one-fifth of that at 4 days (2) but the organelles are separated in the gradient and appear to be intact. Citrate synthetase is present in both mitochondria and glyoxysomes but in very low activity in the soluble region of the gradient, and malate synthetase is absent from mitochondria and again not solubilized from glyoxysomes during preparation. In contrast to the situation in 4-day material when glyoxysomes are fully developed, there are well marked peaks of both synthetases in the region of the gradient occupied by the ER, marked by the activity of NADPH Cyt *c* reductase at density 1.12 g/cc (Fig. 2, center).

Figure 3 shows the changing pattern of activity of malate synthetase in the various organelle fractions at days 2, 3, and 4. At day 2 about 50% of the activity is recovered in the ER, with the remaining activity in the glyoxysomes, and this declines to less than 10%, with >90% in the glyoxysomes by day 4. At no time was there significant malate synthetase activity in other

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[3] Abbreviation: ER: endoplasmic reticulum.

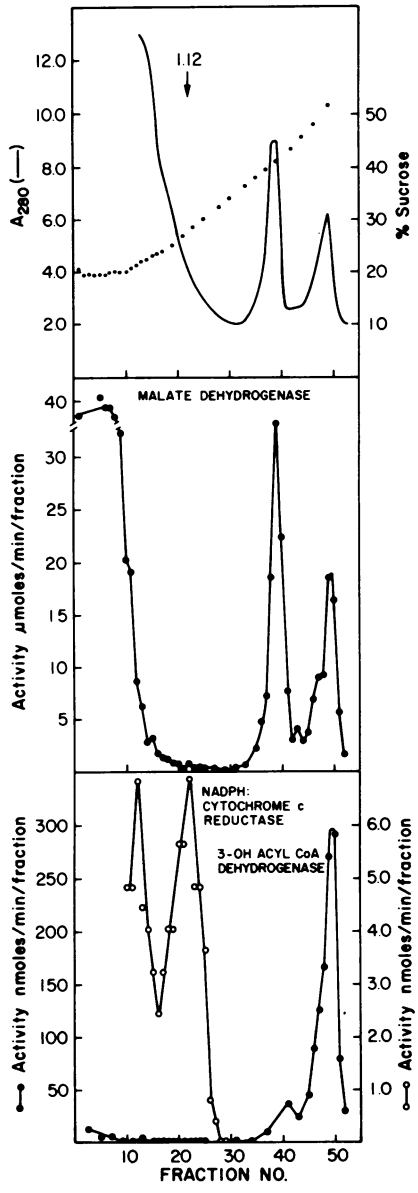

FIG. 1. Protein and enzyme distribution after sucrose gradient centrifugation of homogenates of 4-day endosperm. Markers indicate the position of the ER, the mitochondria, and the glyoxysomes. Fractions (0.6 ml) were collected.

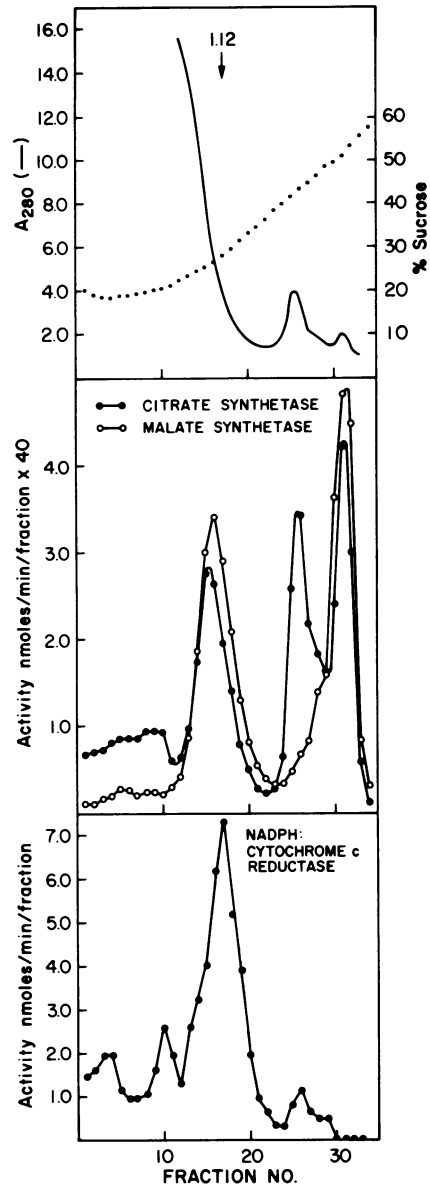

FIG. 2. Protein and enzyme distribution after sucrose gradient centrifugation of homogenates of 2-day endosperm. Markers indicate the position of the ER membranes, the mitochondria and the glyoxysomes. Fractions (1.2 ml) were collected.

regions of the gradient. The relative amount of malate synthetase activity recovered in the ER is highest at the onset of rapid production of glyoxysomes and reaches a low level when glyoxysomal synthesis is complete. A similar conclusion can be made for citrate synthetase (Fig. 3, center), although here the relative activity of the citrate synthetase isoenzyme in the mitochondria also increases strikingly over the same period.

Catalase presents an apparently different developmental picture (Fig. 3, lower). As for the other distinctive enzymes, the glyoxysomes contain almost all of the activity at day 4. At earlier stages, although the ER contains measurable activity, there is no peak in this region of the gradient and the rest of the enzyme is recovered as an apparently soluble component. The changes in the proportion of soluble catalase to glyoxysomal catalase during the 2 to 4 day period are quite similar to the changes in the proportion of ER-associated to glyoxysomal synthetases.

It was shown earlier that, whereas catalase is easily solubilized from glyoxysomes, malate synthetase and citrate synthetase are among those enzymes that are recovered with glyoxysomal membranes when these organelles are deliberately broken by osmotic shock (2, 5). The latter enzymes were, however, released from the glyoxysomal ghosts by treatment with 0.2 M KCl. Examination of the ER portion from 2-day endosperm showed that here, also, a KCl concentration of greater than 0.15 M was required to solubilize > 80% of the activity of the two synthetases.

The behavior of glyoxysomal malate synthetase and citrate synthetase is consistent with their adjunction to the ER prior to vesiculation and formation of glyoxysomes. Since, in contrast to these enzymes, catalase is present exclusively in the lumen of the finished glyoxysomes, it is tempting to suggest that the catalase activity found in the supernatant fraction at early stages may have been present in the ER channels *in vivo* and was released when fragmentation occurred during preparation of the extracts. The behavior of catalase may not, in fact, be inconsistent with its originating in the ER also.

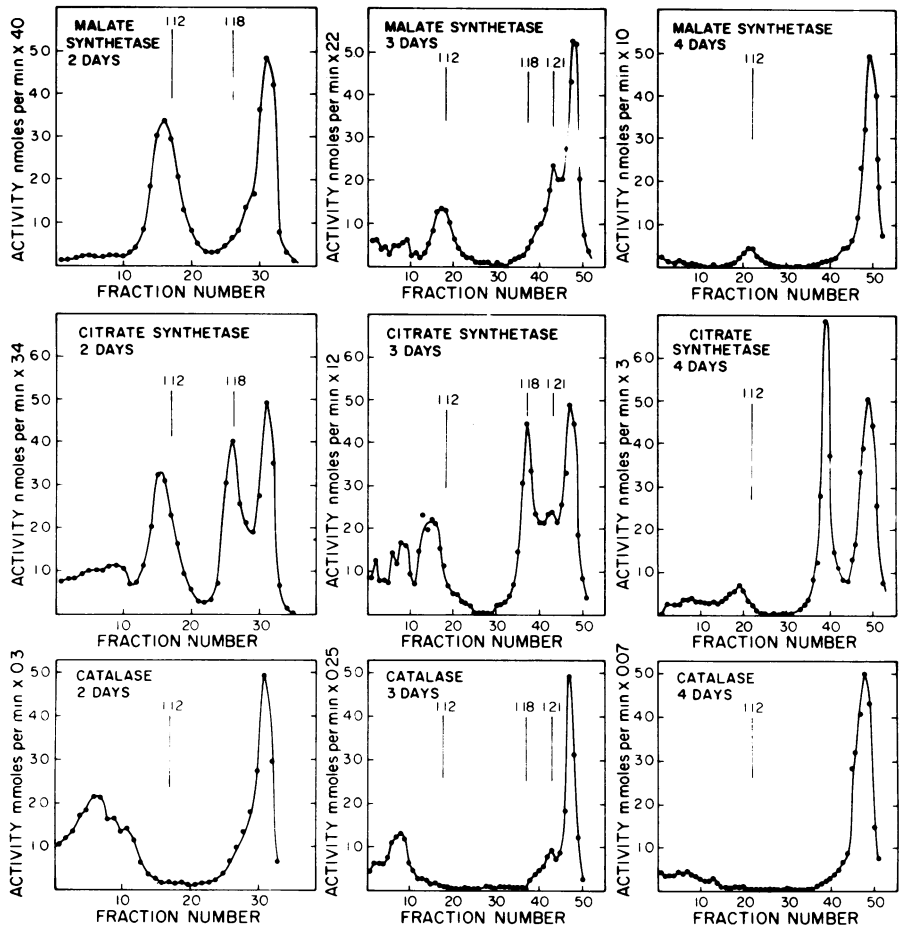

FIG. 3. Enzyme distribution after sucrose gradient centrifugation of 2-, 3-, and 4-day tissue homogenates. The ER membranes band at a peak mean density of 1.12 g/cm³; mitochondria at 1.18 g/cm³, and glyoxysomes at 1.24 g/cm³.

Gel Diffusion Analysis. The presence of proteins in the ER fraction that are in identity with or closely related to glyoxysomal proteins has also been established by double diffusion analysis on Ouchterlony plates. When challenged with the antiserum produced in response to the injection of mixed glyoxysomal proteins (6) the peak glyoxysomal fraction from 5-day endosperm yielded at least three precipitin lines (Fig. 4b). A similar challenge to the ER portion from 2-day endosperm showed that this too contained antigenic components in apparent partial identity to the 5-day glyoxysomal proteins (Fig. 4b). Figure 4a shows that 2-day ER and glyoxysomes have a common antigenic constituent, and from Figure 4b it is clear that this antigen is present in membrane pellets prepared from the ER. Antigenic materials are also present in the soluble regions of the gradient but mitochondrial fractions yielded completely negative results (data not shown).

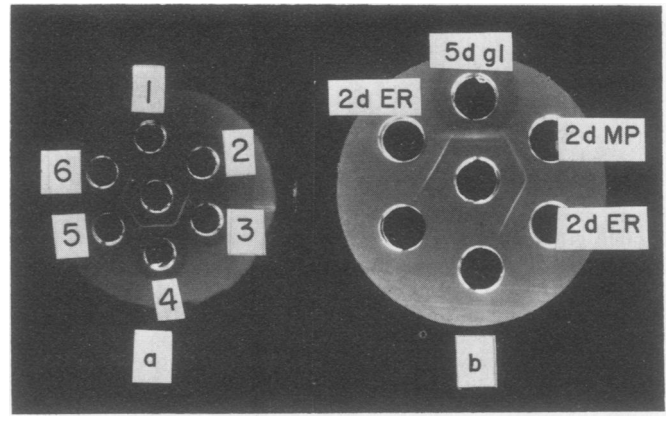

FIG. 4. Photographs of precipitin band patterns in Ouchterlony plates after challenges of various samples with antiglyoxysome antiserum (in the center wells). a: Reactivity of 20λ of the peak fraction of 2-day glyoxysomes (1 and 2, seen as diffuse, faint bands) and 20λ of the peak fraction of 2-day ER membranes (3 to 6); b: reactivity of 20λ of 5-day glyoxysome peak fraction (5d gl). 20λ of a resuspended membrane pellet (post dilution and 100,000g spin), and 20λ of 2-day ER peak fractions (2d ER).

DISCUSSION

This report provides additional biochemical evidence for the involvement of the ER in the formation of glyoxysomes. This relationship has been previously suggested by electron microscope studies (14), immunocomparisons of membrane antigens (4), and studies of biosynthesis of membrane phospholipids (7, 9, 10, 12). Related data have been adduced as evidence for a similar relationship between ER and leaf peroxisomes (8).

Particular significance was attached to the observation that two of the key glyoxysomal enzymes, malate synthetase and citrate synthetase, are found in relatively high activity in the ER at early stages of germination, suggesting that enzymatic constituents of the glyoxysomes, as well as membrane phospholipids, are derived fairly directly from the ER. This implication denies a trivial explanation of the results, namely that the enzymic activities measured in the ER fractions arose from broken glyoxysomes. The main argument against such an explanation is that

when glyoxysomes are deliberately broken by osmotic treatment (5) they give rise to ghosts which have an equilibrium density of 1.21 g/cc, and are thus recovered in the gradient (with associated malate synthetase and citrate synthetase) well below the ER (5).

Immunodiffusion assays also reveal the presence of other proteins, whose function remains unknown but which are recognized by the antiglyoxysome antiserum, and are also found on the ER. The significance of the presence of a strong antigenic response in the soluble region of the gradient is not clear. Since, in the finished glyoxysome, most of the proteins are not associated with the membrane (5), it seems reasonable to suggest, as was argued for catalase, that they are released from the ER during the extraction procedure, and thus appear in the soluble region of the gradient.

The details of the packaging sequence are still largely unknown. The data presented here do not suggest a precursor particle of lower buoyant density as was found in developing leaves (1). We also see no dramatic change in the ratio of malate synthetase-citrate synthetase-catalase in the glyoxysome peak for the three stages studied. If glyoxysomes increase in size during a "maturing" process, it is likely that enzymes are added to the organelle in constant or nearly constant proportions.

The regulation and precision of glyoxysome formation and enzyme packaging in castor bean endosperm make it a useful system for the study of more basic questions about the role of membranes in polysome deployment and routing of extracytosolic proteins within the cell.

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