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Organelle-Specific Isozymes of Citrate Synthase in the Endosperm of Developing *Ricinus* Seedlings[1]

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ABSTRACT

Chromatographic analysis of organelle-associated citrate synthase activity revealed distinct mitochondrial and glyoxysomal forms of the enzyme. The chromatographic elution patterns on hydroxylapatite, carboxymethylcellulose and DEAE-cellulose of citrate synthase from the endosperm of 4.5-day-old castor bean seedlings revealed significant differences for mitochondrial and glyoxysomal activities of the enzyme. The endoplasmic reticulum-associated citrate synthase activity eluted from DEAE-cellulose in a pattern that was identical to that of the glyoxysomal activity. The same kinds of organelle specific isozyme elution patterns were observed with young, developing seedlings. Gibberellic acid-treatment of young seedlings increased total recoverable citrate synthase activity from endosperm tissue but did not modify the organelle specific isozyme relationships.

One of the major concerns of our laboratory is the question of how glyoxysomes are assembled during the early stages of castor bean germination (14, 15). In particular, we are interested in the role of ER membranes in glyoxysomal assembly and the role of gibberellins in accelerating and regulating fine-tuned expression of glyoxysomal function.

During early stages of castor bean germination, cellular organelles rapidly proliferate in endosperm tissue (13, 15, 21, 34, 36). How these organelles acquire their constituent proteins poses an interesting developmental problem. In the case of glyoxysomal biogenesis, glyoxysomes are thought to arise from ER through a budding process (3, 16, 18, 35, 36). Glyoxysomal proteins are thought to be synthesized by ER bound ribosomes and mobilized across ER membranes into cisternae where they accumulate within budding precursors of glyoxysomes (3, 16, 18, 35, 36). Recent elaboration of the "signal hypothesis" (4, 5) provides a plausible mechanism to describe how some proteins are transferred across membranes into developing organelles.

The following reasons are cited for believing that the ER plays a central role in glyoxysomal development in castor bean: ER is the primary site of synthesis of glyoxysomal membrane lipids (3, 6, 21, 23, 24, 31, 32); ER and glyoxysomal membranes have many similar (or identical) proteins as revealed by SDS-polyacrylamide gel electrophoresis (7–9); a precursor-product relationship between ER and glyoxysome was demonstrated by protein labeling studies (7, 22); and ER serves to catalyze glycosylation of glyoxysomal proteins (28–30). Mitochondria and proplastids also proliferate in a developmental pattern similar to glyoxysomes in castor bean endosperm (13, 21, 26) and in other germinating fatty seedlings (11, 12, 16, 20, 34, 35). Although it is clear that the ER functions to provide phospholipids for general organelle biogenesis in castor bean endosperm (21, 23, 24, 31, 32), possibilities of its involvement in protein synthesis and subsequent transmembrane transport of mitochondrial and proplastid proteins have largely been overlooked.

A suggestion that ER may play a role in the mobilization of proteins into mitochondria can be inferred from the observation that a significant fraction of the citrate synthase activity is compartmented with the ER during early stages of castor bean germination (15). However, organellar identity of this citrate synthase activity is not yet established.

Limited evidence indicates that glyoxysomal and mitochondrial enzymes are isozymes distinct from one another. Axelrod and Beevers (1) reported inhibition of only the mitochondrial activity by ATP. In contrast, Huang *et al.* (19) have provided evidence for the inhibition of both enzymes by ATP, and for immunological similarity of the respective organellar activities. Barbareschi *et al.* (2) have shown differences in heat sensitivity, pH dependence of activity, inhibition by ATP, and molecular sieving properties for the mitochondrial and glyoxysomal citrate synthase activities from maize scutellum.

In this paper, we provide evidence that the citrate synthase activities of mitochondria and glyoxysomes represent distinct isozymes and that the activity found to be associated with the ER is chromatographically identical to the glyoxysomal isozyme. We also illustrate GA_3-stimulated accumulation of citrate synthase in both mitochondria and glyoxysomes in developing endosperm and show citrate synthase activity associated with ER to be identical to the glyoxysomal form. Possible consequences of these observations will be discussed in terms of the synthesis and incorporation of citrate synthase into glyoxysomes and mitochondria.

MATERIALS AND METHODS

Seeds of castor bean (*Ricinus communis*, cv. Hale; McNair Seed Co., Plainview, TX) were soaked in running tap water for 24 h. The imbibed seeds were transferred to moist vermiculite and incubated at 30 C in darkness in a Percival (Boone, IA) germinator. RH was maintained at 90%.

Seedling Selection and Hormone Treatment. Seedlings were selected for GA_3 treatment as previously described (14). After 48 h germination, a uniform class of seedlings having radicles between 2 and 5 mm long were selected. These seedlings were surface sterilized with 5% commercial NaOCl for 5 min and rinsed thoroughly in sterile distilled H_2O. Seedlings, in groups of 10, were placed on cheesecloth (seven layers) in 10-cm diameter Petri

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plates. Seedlings were covered with two layers of cheesecloth and 5 ml of treatment solution were added. The Petri plates were covered and incubated at 30 C for 10 or 20 h. The treatment solutions were, respectively, controls consisting of (a) sterile distilled H_2O, and (b) sterile 70 μM GA_3 solution. In addition to the 2-day-old seedlings used for hormone treatment, a uniform population of 4.5-day-old seedlings germinated in vermiculite (21) were harvested for comparative purposes.

Purification of Organelles from Endosperm Tissue. Endosperm tissue from 2-day-old hormone treated seedlings and the respective water treated control and from 4.5-day-old seedlings were harvested for homogenization and isolation of organelles. Endosperm from 10 seedlings of each treatment was homogenized as previously described (15, 24). The grinding medium contained 150 mM Tricine buffer (pH 7.6), 0.5 M sucrose, 10 mM KCl and 1 mM EDTA.

Organelles contained in the crude extract were purified by sucrose density gradient fractionation (15, 24). Crude organelle preparations were centrifuged for 4 h at 80,000*g* using a Beckman SW 25.2 rotor in a Beckman L-2 preparative ultracentrifuge. Gradients were fractionated as previously described (1.2 ml per fraction) (14, 15). Gradient fractions were assayed for citrate synthase activity and fractions representing, respectively, purified ER, mitochondria, and glyoxysomes were combined.

Extraction of Citrate Synthase Activity from Purified Organelles. Purified mitochondria and glyoxysomes were subjected to osmotic shock by dilution with an equal volume of 50 mM Tricine buffer (pH 7.6) containing 1 mM EDTA over a 5 min period with gentle mixing. Purified ER was diluted with buffer (3/1, v/v). The osmotically shocked organelles were centrifuged at 40,000*g* for 30 min. Citrate synthase activity was extracted from the organelle pellets by resuspending the respective pellets in 50 mM Tricine buffer (pH 7.6) containing 200 mM KCl, 1 mM EDTA and 0.2% (v/v) Triton X-100. After 1 h with occasional stirring, the suspension was centrifuged at 40,000*g* for 60 min. The supernatant was saved and the pellet extracted two more times with the same buffer. The three extracts were combined and dialyzed extensively against 5 mM phosphate buffer (K^+ salt, pH 7.6). Dialyzed samples were cleared of any residue by centrifugation at 10,000*g* for 30 min. The clarified extract was then applied to hydroxylapatite, DEAE-cellulose or carboxymethyl-cellulose and eluted as described below.

Chromatographic Fractionation of Citrate Synthase Activity. In the first sets of experiments (Figs. 1–3), 3 ml of enzyme extract from purified mitochondrial or glyoxysomal fractions derived from 4.5-day-old seedlings were applied to the appropriate chromatography medium contained in a 1.5-x 3-cm column (about 5 ml bed volume) that had been preequilibrated with 5 mM phosphate buffer (K^+ salt, pH 7.6). After the sample had been applied by gravity, citrate synthase activity was eluted from the column. For hydroxylapatite (HT from Bio-Rad, Richmond, CA) and DEAE-cellulose (DE-52 from Whatman, Clifton, NJ), the enzyme was eluted stepwise with 12 ml each of the following phosphate buffers: (K^+ salt, pH 7.6) 5, 25, 50, 75, 100, 150, 200 and 500 mM. For carboxymethyl-cellulose (CM-cellulose, fine grade, from Sigma), citrate synthase was eluted stepwise with 12 ml each of the following phosphate buffers: (K^+ salt, pH 7.6) 5, 10, 20, 25 and 500 mM. One to three enzyme units (μmol/min) of citrate synthase activity containing 0.5 to 3 mg protein were applied to the chromatographic medium for fractionation and the columns were eluted by gravity. Fractions of about 2 ml were collected with an ISCO fractionator equipped with a UA5-UV monitor. In these experiments, recovery of citrate synthase activity was >70%. All procedures, except column elution, were carried out in the cold (4 C). Column chromatography was carried out at room temperature (24 C).

In the second set of experiments (Figs. 5–7), enzyme preparations from 2-day water- and GA_3-treated seedlings, and from 4.5-day-old seedlings (2 ml of enzyme containing 0.5 to 2 units of activity and 1 to 3 mg protein) were applied by peristaltic pumping (flow rate of 0.3 to 0.5 ml/min) to a 1.6- × 5-cm Pharmacia column (10 ml bed volume) of DEAE-cellulose (DE-52 from Whatman) preequilibrated with 5 mM phosphate buffer (K^+ salt, pH 7.6). The enzyme was stepwise eluted with continuous pumping with 25 ml each of the following phosphate buffers: (K^+ salt, pH 7.6) 5, 25, 50, 75, 100, 150, 200, 300 and 500 mM at a rate of 0.3 to 0.5 ml/min. Fractions of about 1 ml were collected with the ISCO fractionator. All procedures except chromatographic fractionation of citrate synthase preparations were carried out at 4 C. Chromatography was carried out at room temperature (24 C). In this set of experiments, more than 90% of the citrate synthase activity applied to the columns was recovered.

Assays. Citrate synthase was assayed as previously described (15, 17). Protein was measured after the method of Lowry *et al.* (25).

Chemicals. Reagent grade chemicals were used. Biochemicals including GA_3, grade III were obtained from Sigma.

RESULTS

Chromatography of Citrate Synthase Activity from Purified Mitochondria and Glyoxysomes. Consistent with our previous studies, glyoxysomes attained their highest functional activities in the developing endosperm between 4.5 to 5 days of seedling germination (21). The rise and fall of citrate synthase activity paralleled this developmental pattern; thus 4.5-day-old seedlings were selected as material to examine isozymic relationships between mitochondrial and glyoxysomal citrate synthase activities. Enzyme extracts were prepared from purified mitochondria and glyoxysomes and chromatographed on hydroxylapatite, carboxymethyl-cellulose, or DEAE-cellulose media. The elution pattern of the mitochondrial activity differed significantly from the glyoxysomal activity on each of the three chromatographic media used. Figure 1 shows the stepwise elution pattern of mitochondrial and

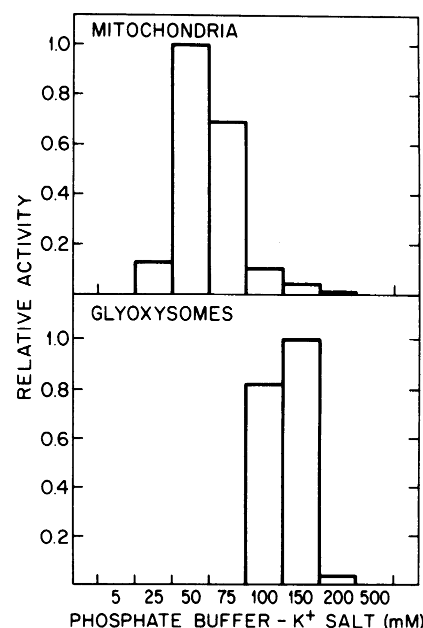

FIG. 1. Fractionation on hydroxylapatite of citrate synthase activity from extracts of purified mitochondria and glyoxysomes derived from endosperm of 4.5-day-old seedlings. Details of enzyme preparation and chromatography are described in "Materials and Methods." A relative unit of 1.0 is equivalent to: mitochondria, 1.65 μmol/min; glyoxysomes, 1.59 μmol/min.

glyoxysomal citrate synthase activity from hydroxylapatite. Most mitochondrial activity was eluted with 50 and 75 mM phosphate buffer, whereas the glyoxysomal activity was eluted with 100 and 150 mM phosphate. On carboxymethyl-cellulose (Fig 2), mitochondrial citrate synthase did not bind and eluted in the void volume. However, the glyoxysomal citrate synthase bound to carboxymethyl-cellulose and most was eluted with 15 mM phosphate buffer (K^+ concentration was 28 mM). On DEAE-cellulose (Fig. 3), the mitochondrial enzyme was strongly bound to the ion exchange matrix and most eluted with the 75, 100 and 150 mM phosphate ion steps. The glyoxysomal enzyme was less strongly bound to DEAE-cellulose and most of the adsorbed activity eluted with 25 mM phosphate buffer.

In 4.5-day-old seedlings, a small amount of citrate synthase activity is associated with the ER membrane fraction (Fig. 3 in Ref. 15). We prepared this ER-associated activity for chromatography and the results of DEAE-cellulose chromatography are shown in Figure 3. The ER-associated activity eluted in a pattern that closely paralleled the glyoxysomal isozyme, indicating strong similarity between the ER and glyoxysomal citrate synthases.

Effect of GA_3-Treatment on the Early Development of Citrate Synthase Activity. Association of the glyoxysomal-type citrate synthase with ER membranes is analogous to the association of malate synthase and other glyoxysomal enzymes to these membranes in 4.5-day old seedlings (15, 22). Because ER association of glyoxysomal enzyme activities is most dramatically expressed in the endosperm of 2-day-old seedlings (15), it was of interest to determine whether any of the mitochondrial isoenzyme could be found associated with the ER at this early stage. Since GA_3 treatment of seedlings both accelerates germination and increases the amount of glyoxysomal malate synthase activity in endosperm of 2-day-old seedlings (14, 37), questions are raised as to whether GA_3 altered the composition of the citrate synthase isozymes associated with the ER.

Application of GA_3 did not significantly influence development of citrate synthase activity in the mitochondrial, glyoxysomal or ER fractions during the first 10 h of GA_3 treatment (Table I).

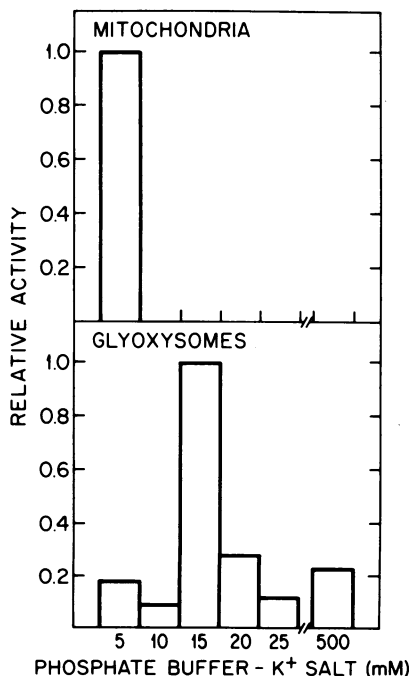

FIG. 2. Fractionation on carboxymethyl-cellulose of citrate synthase activity from extracts of purified mitochondria and glyoxysomes derived from endosperm of 4.5-day-old seedlings. A relative unit of 1.0 is equivalent to: mitochondria, 2.9 μmol/min; glyoxysomes, 1.18 μmol/min.

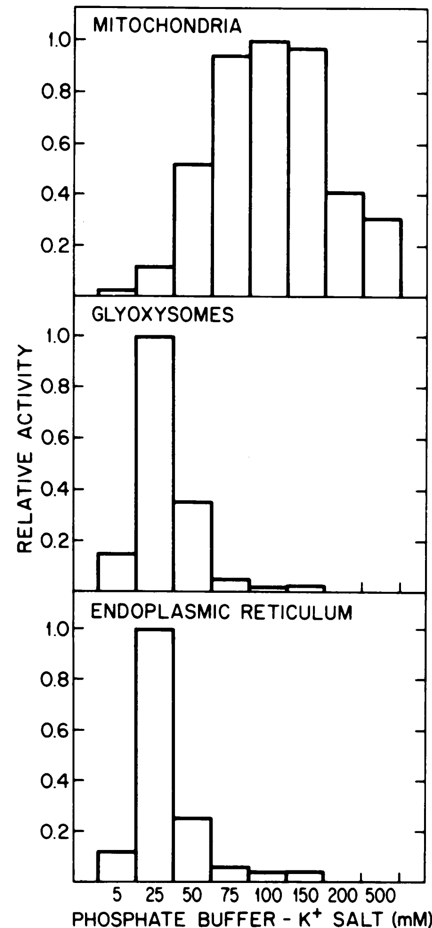

FIG. 3. Fractionation on DEAE cellulose of citrate synthase activity from extracts of purified mitochondria, glyoxysomes and ER derived from endosperm of 4.5-day-old seedlings. A relative unit of 1.0 is equivalent to: mitochondria, 0.80 μmol/min; glyoxysomes; 1.74 μmol/min; ER, 0.38 μmol/min.

After 20 h, GA_3 treatment had significantly increased enzyme activity in mitochondria and glyoxysomes; however, no significant increase in the amount of citrate synthase associated with the ER could be observed in the GA_3-treated seedlings. Figure 4 compares citrate synthase activity associated with various organelle fractions recovered by sucrose density gradient fractionation of the organelles contained in crude extracts from the GA_3-treated and control seedlings.

DEAE-Cellulose Chromatography of Citrate Synthase Activities from Mitochondria, Glyoxysomes and ER of GA_3-Treated and Control Seedlings. Since GA_3 treatment of 2-day-old seedlings increased the amount of citrate synthase that accumulated in mitochondria and glyoxysomes, it was of interest to determine whether GA_3 treatment had any influence in altering the form of the enzyme associated with the ER during early stages of seedling development.

The elution patterns of the mitochondrial activities recovered from 2 day + 20 h GA_3-treated seedlings and the 2-day + 20 h control seedlings are compared (Fig. 5). The elution pattern of mitochondrial activity for 4.5-day-old seedlings is included as a second control to show how mitochondrial citrate synthase from fully active endosperm tissue elutes from DEAE-cellulose. In all three examples, the mitochondrial activity predominantly elutes with 75 and 100 mM phosphate buffer; the overall elution pattern of these three examples is essentially identical. Comparing the DEAE chromatographic patterns of the various organellar citrate synthases from the 4.5-day old seedlings (Figs. 5-7) with our

Table I. *The Effect of GA_3 Treatment on the Development of Citrate Synthase Activities Associated with Mitochondria, Glyoxysomes and ER in the Endosperm of 2-Day-Old Castor Bean Seedlings*

Length of GA_3 Treatment	Mitochondria		Glyoxysomes		ER	
	Control	GA_3	Control	GA_3	Control	GA_3
h			*% of control*			
0	100 (0.50)[a]		100 (0.40)			
10	100 (2.10)	105	100 (1.25)	100		
20	100 (3.50)	130	100 (1.78)	205	100 (0.82)	107

[a] Values in parentheses indicate citrate synthase activities recovered: μmol/min 20 endosperm halves.

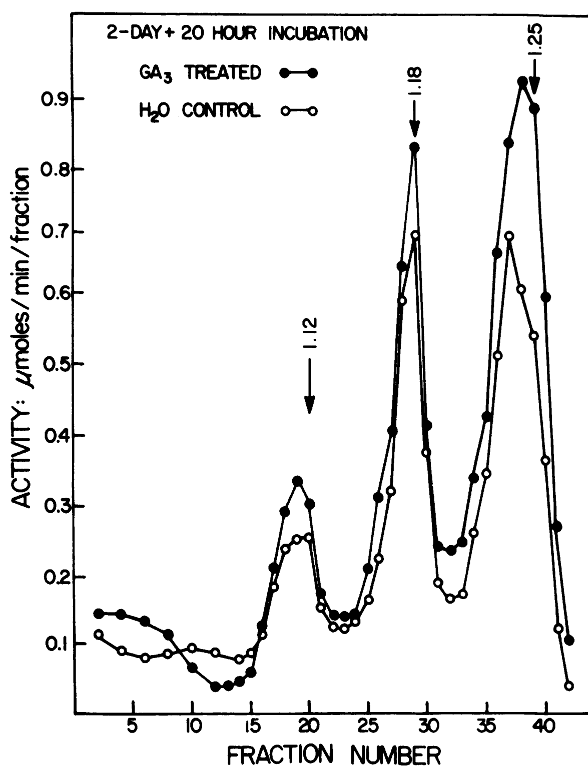

FIG. 4. Distribution of citrate synthase activity after organelle separation by sucrose density gradient centrifugation of homogenates from 20 endosperm halves.

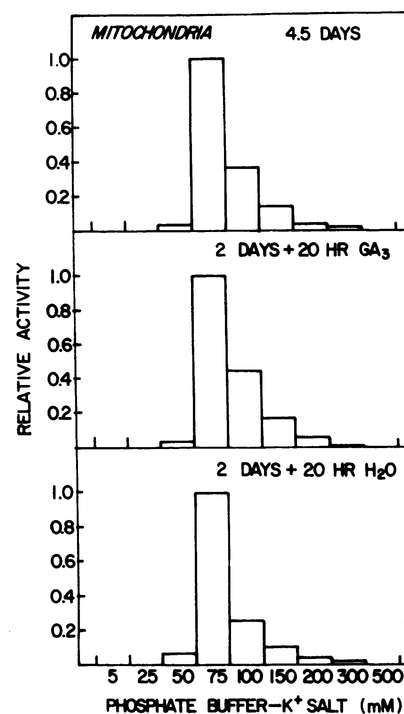

FIG. 5. Fractionation on DEAE cellulose of citrate synthase activity of purified mitochondria from 4.5-day-old seedlings, 2-day-old + 20 h GA_3-treated seedlings, and 2-day-old + 20 h H_2O-treated seedlings. A relative activity of 1.0 is equivalent to: 4.5 days, 1.80 μmol/min; 2 days + 20 h GA_3, 1.63 μmol/min; 2 days + 20 h H_2O, 1.52 μmol/min.

previous results (Fig. 3) reveals slightly altered elution patterns. Most of the glyoxysomal and ER activities elute with 25 mM phosphate buffer. The mitochondrial isozyme (Fig. 5) elutes at a lower phosphate concentration (75 and 100 mM) compared to that seen in Figure 3. These altered elution patterns are probably due to the smaller amounts of protein and enzyme activity applied to the ion exchange resin, to a greater bed volume of DEAE-cellulose, and to the continuous and steady peristaltic pumping of the elution buffers (Figs. 5–7) rather than elution by gravity (Fig. 3).

Figures 6 and 7 show, respectively, the elution of glyoxysomal and ER enzymes from 4.5-day-old seedlings, from 2 day + 20 h GA_3-treated, and from 2 day + 20 h control seedlings it is clear that citrate synthase activities from the above samples have nearly identical elution patterns. These results indicate that citrate synthase isozymes from young and from fully developed endosperm are essentially identical and that GA_3 treatment does not result in an altered pattern of association of the mitochondrial isozyme with the ER.

DISCUSSION

There is now considerable evidence to support the notion that ER plays a role in the biogenesis of glyoxysomes. On the other hand, except for studies that show the ER as a major site of phospholipid synthesis for mitochondrial membrane development (3, 21, 23, 24, 31, 32), little substantive evidence is available to implicate the ER in the process of incorporation of proteins into mitochondria during its assembly. Mitochondria and glyoxysomes have activities of several enzymes in common; examples include malate dehydrogenase, citrate synthase, and aconitase. In an earlier study, González and Beevers (15) showed a considerable fraction of citrate synthase activity associated with the ER fraction in young endosperm tissue of castor beans. This association with ER was like that of malate synthase, which is one of the unique, functional, marker enzymes of glyoxysomes. This observation raised questions of whether activities associated with the ER represented both mitochondrial and glyoxysomal forms, and whether mitochondrial and glyoxysomal citrate synthases were, in fact, distinct isozymes. Moreover, González (14) and Wrigley and Lord (37) showed that exogenously applied GA_3 stimulated the development of several ER and glyoxysomal marker enzyme activities during early phases of seed germination. Thus, it was of interest to determine whether GA_3 also influenced changes in the development of mitochondrial, glyoxysomal, and ER-associated citrate synthases.

The present results show that the glyoxysomal and mitochon-

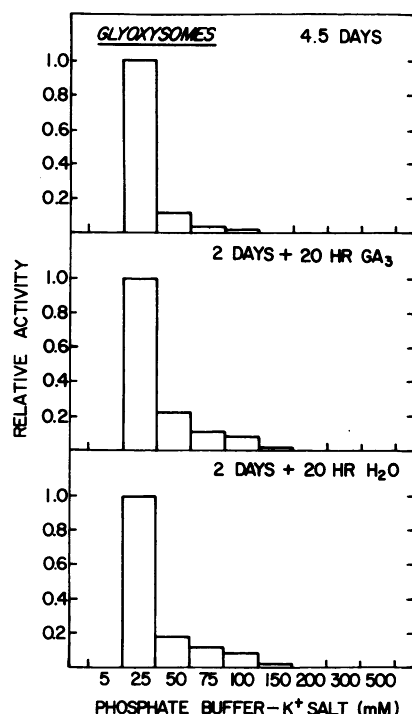

FIG. 6. Fractionation on DEAE cellulose of citrate synthase activity from extracts of purified glyoxysomes from 4.5-day-old seedlings, 2-day-old + 20 h GA_3-treated seedlings, and 2-day-old + 20 h H_2O-treated seedlings. A relative activity of 1.0 is equivalent to: 4.5 days, 1.11 μmol/min; 2 days + 20 h GA_3, 0.95 μmol/min; 2 days + 20 h H_2O, 0.77 μmol/min.

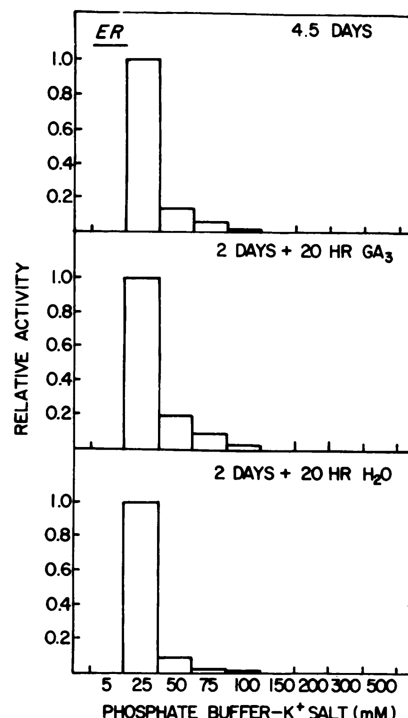

FIG. 7. Fractionation of DEAE cellulose of citrate synthase activity from extracts of purified ER from 4.5-day-old seedlings, 2 day + 20 h GA_3-treated seedlings, and 2-day-old + 20 h H_2O-treated seedlings. A relative activity of 1.0 is equivalent to: 4.5 days, 0.96 μmol/min; 2 days + 20 h GA_3, 0.56 μmol/min; 2 days + 20 h H_2O, 0.77 μmol/min.

drial citrate synthases represent two chromatographically distinct classes of enzymes and that the ER-associated activity is identical to the glyoxysomal isozyme in its chromatographic behavior on DEAE cellulose. These relationships are found in seedlings during the early development of the endosperm, after GA_3 induced changes in endosperm development and in the metabolically most active endosperm tissue (from 4.5-day-old seedlings). This association of the glyoxysomal isozyme of citrate synthase with the ER fraction is consistent with previous results indicating a role for ER in glyoxysomal biogenesis (10, 15, 22). While these results do not exclude the participation of ER in the incorporation of citrate synthase into developing mitochondria, it is reasonable to conclude that the sequence of events that lead to the synthesis and assembly of citrate synthase in mitochondria differs from that in glyoxysomes.

By way of speculation, operationally there are several sites in the sequence of events leading to the biogenesis of an organelle that can provide for selective incorporation of a particular isozyme into its respective compartment: (a) transcription of the respective messenger RNA's for separate isozymes may occur at temporally distinct periods of time. This kind of message processing can provide a developmental mechanism for differential genesis of two separate organelle populations. There is presently no indication of this kind of development in castor bean endosperm. (b) Translation of one isozyme may take place on membrane bound ribosomes whereas the synthesis of the other isozyme(s) may occur via the soluble or cytosolic polysomal system. (c) Further discrimination can occur at the receptor site for the isozyme (or with the recognition of a putative "signal sequence" on the isozyme) on the ER membranes destined for glyoxysomal development or on the outer mitochondrial membrane system involved in this organelle's growth and development. (d) Another model that can provide for fitting an isozyme into its appropriate organelle may be the postmodification events such as glycosylation or proteolytic cleavage and resultant activation of the proenzyme. For example, it has been shown that many glyoxysomal proteins (26, 27, 33) including malate synthase (26) are glycosylated, and that protein glycosylation takes place on ER membranes (28–30). Whether the glyoxysomal citrate synthase likewise is glycosylated and thereby different from its mitochondrial counterpart remains to be seen. The postmodification processes raise the interesting possibility that incorrect modification will result in a dysfunctional enzyme and that this dysfunctional form is rapidly and selectively destroyed. One or all of these possibilities may be in operation to provide the cell with a sequential editing system that maintains a high degree of fidelity in the compartmentation of isozymes into their respective organellar loci.

We are currently extending our studies to identify the sites of synthesis of glyoxysomal and mitochondrial citrate synthases and to the purification and detailed analyses of the properties of these two isozymes.

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