ASSOCIATION OF THE GLYOXYLATE CYCLE ENZYMES IN A NOVEL SUBCELLULAR PARTICLE FROM CASTOR BEAN ENDOSPERM

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Received April 13, 1967

The reactions of the glyoxylate cycle play an essential role in the massive conversion of storage lipid to sucrose during the germination of fatty seeds such as castor bean (Kornberg and Beevers, 1957; Canvin and Beevers, 1961). Previous studies of the intracellular distribution of malate synthetase and isocitrate lyase in plant tissues carrying out this conversion have demonstrated that significant proportions of these enzymes sediment with the mitochondria (Marcus and Velasco, 1960; Tanner and Beevers, 1965). Since, in addition, the enzymes catalyzing reactions common both to the TCA cycle and the glyoxylate cycle are believed to be mitochondrial, the view has grown that *in vivo* the glyoxylate cycle functions in these organelles.

Treatment of the crude mitochondrial fraction on sucrose density gradients has now revealed that enzymes of the glyoxylate cycle are not present in the purified mitochondria; they are specifically associated with a distinct particulate component of higher density. This component will be referred to as glyoxysomes.

MATERIALS AND METHODS

Materials:

Castor bean (*Ricinus communis* L.) variety Baker 296 were a kind gift

from Castor Oil Company, Plainview, Texas. The seeds were soaked for approximately twelve hours and then germinated for five days in moist vermiculite at 30°C. The testae, roots, hypocotyl and cotyledons were removed and the endosperm tissue used as starting material.

Methods:

Endosperm tissue (25 g) was homogenized in 100 ml of 0.4 M sucrose, 0.05 M Tris buffer pH 7.5, 0.01 M dithiothreotol, 0.1% bovine serum albumin, 0.01 M KCl, 1×10^{-3} M EDTA, 1×10^{-4} M $MgCl_2$. The tissue was first chopped in a household onion chopper and then vigorously ground for 30 seconds with a mortar and pestle. No abrasive was used. The resulting brei was filtered through cheesecloth and centrifuged at 500 x g for 10 minutes. The supernatant solution was centrifuged at 10,000 g for 10 minutes and the resulting pellet resuspended in 4 to 6 ml of 32% sucrose. Homogenates prepared in this manner gave significantly higher yields of isocitrate lyase in the pellets than had previously been obtained. Portions of this crude particulate suspension containing 15 to 20 mg of protein were then layered on linear sucrose gradients contained in Spinco rotor SW 25-2 cellulose nitrate tubes. The slope of the linear gradients was tripartite, consisting of 16 ml of 60% sucrose, 13 ml grading from 60% to 45% sucrose and 26 ml grading from 45% to 32% sucrose.

The gradients were centrifuged a minimum of four hours at 23,000 r.p.m. in a Spinco L-2-50 ultra-centrifuge and allowed to decelerate without braking. The gradients were fractionated into thirty-five 1 ml fractions numbered consecutively in the order of increasing density. Fractions were maintained at 0-5°C and assayed as quickly as possible.

All enzymes were assayed optically with a dual beam spectrophotometer equipped with a recorder. Isocitrate lyase was assayed by the method of Dixon and Kornberg (1959); fumarase and aconitase by the method of Racker (1950); citrate synthetase at pH 8.0 by the method of Srere *et al.* (1963); malate synthetase at pH 8.0 by the method of Hoch and Beevers (1966); NADH

oxidase by the method of Crane (1957); NAD isocitric dehydrogenase by the method of Kornberg (1955); NADP isocitric dehydrogenase by the method of Ochoa (1955a) and malic dehydrogenase by the method of Ochoa (1955b). All of the above dehydrogenase assays were run in the presence of 3.3 mM KCN. Succinic dehydrogenase was assayed by the method of Hiatt (1961). The enzyme was incubated with succinate for 15 minutes before initiating the reaction with phenazine methosulphate. Cytochromes were detected by means of difference spectra according to the method described previously (Breidenbach et al., 1967). Protein was determined by the method of Lowry et al., 1951).

RESULTS

Four bands are discernible after sucrose density gradient centrifugation of crude particulate suspensions (Figures 1 and 2). The uppermost band (A) consists almost exclusively of mitochondria as demonstrated by enzyme composition (Table I) and electron microscopic examination. The next denser band (B) has been tentatively identified as proplastids on the basis of light, U.V. and electron microscopy. The third band (C) is comprised of the particulates referred to as glyoxysomes in this paper. The fourth and densest band (D) is a very minor protein component and has not been characterized.

Figure 2 illustrates the protein distribution profile for a typical gradient separation. The mitochondrial band and the glyoxysome band contain roughly equivalent amounts of protein and together constitute more than half of the total protein layered on the gradient.

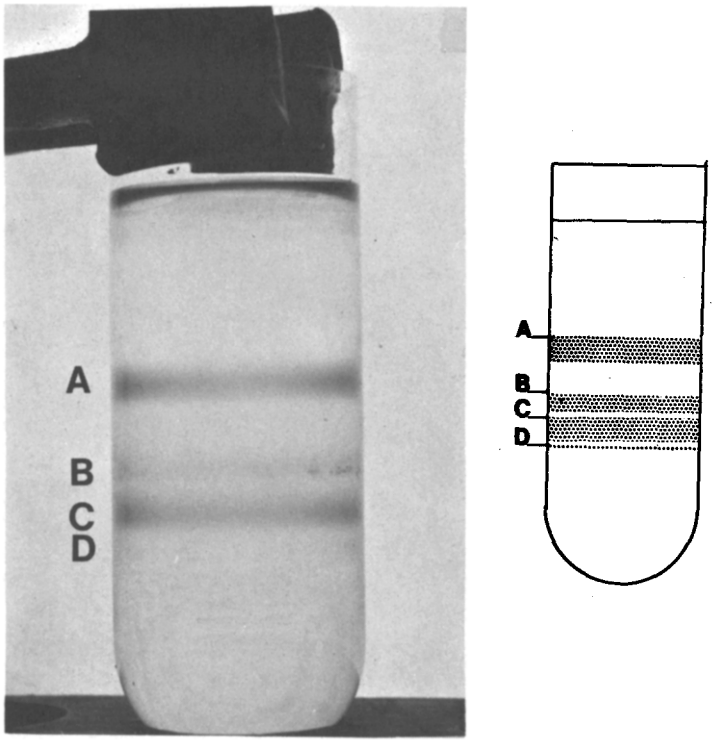

Figure 1. Typical separation of the components of the crude particulate fraction from 5 day old castor bean endosperm on a sucrose density gradient. A. Mitochondria. B. Proplastids. C. Glyoxysomes. D. Unknown component.

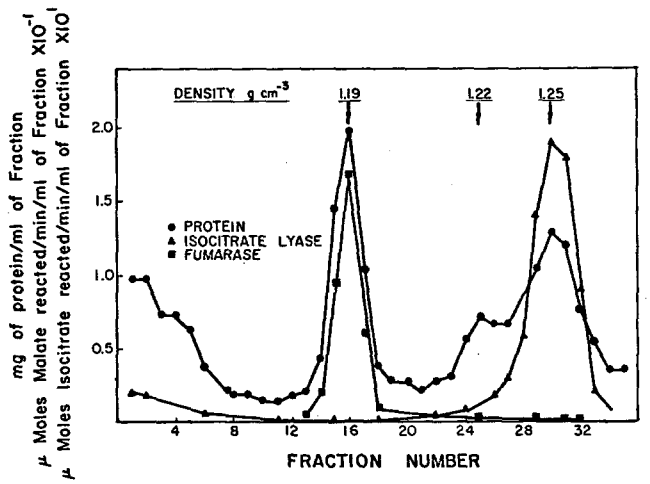

Figure 2. Distribution of protein, fumarase and isocitrate lyase after sucrose density gradient separation of the components of the crude particulate fraction from 5 day old castor bean endosperm.

TABLE I

ENZYME ACTIVITIES IN SUBCELLULAR FRACTIONS

SEPARATED FROM CASTOR BEAN ENDOSPERM

	GLYOXYSOME (Density) 1.25 g cm^{-3}	MITOCHONDRIA (Density) 1.19 g cm^{-3}
	Enzyme Activity (micromoles substrate consumed/min/mg protein)	
Isocitrate lyase	1.16	0
Malate synthetase	1.48	0
Citrate synthetase	1.16	0.72
Malate dehydrogenase (NAD)	22	50
Fumarase	0	1.4
NADH oxidase	0	0.11
Succinic dehydrogenase	0	0.55

The glyoxysome band contains 60-70% of the total isocitrate lyase activity found in the original homogenate. The remainder is about equally distributed between the 500 g pellet and the supernatant solution remaining after sedimenting the crude particulate fraction at 10,000 x g. The distribution of this enzyme on a typical gradient is shown in Figure 2. Clearly the activity is associated with the glyoxysome. Malate synthetase activity shows an identical pattern. Fumarase activity, by contrast, is specifically associated with the mitochondria and is absent from the glyoxysome.

Table I shows the distribution of seven enzyme activities in the separated glyoxysome and mitochondrial fractions. Two of these, isocitrate lyase and malate synthetase are confined to the glyoxysomes and absent from mitochondria. Two, citrate synthetase and malate dehydrogenase are present in both particles. Fumarase, NADH oxidase and succinate dehydrogenase are present in the mitochondria and can not be detected in the glyoxysomes. A further contrast between the two particles is observed in the reduced-oxidized difference spectra. Clear cytochrome peaks were seen in the mitochondrial fraction but none could be detected in the glyoxysomes.

Other enzymes of interest are the isocitrate dehydrogenases and aconitase. Only very low levels of NAD and NADP isocitrate dehydrogenases and aconitase (less than 4% of the isocitrate lyase) were detected in the crude particulate suspension. Considerably greater amounts of NADP isocitrate dehydrogenase and aconitase were present in the original supernatant solution but the aconitase lost activity rapidly even at $0^{\circ}C$.

DISCUSSION

Malate synthetase and isocitrate lyase are specifically associated with a non-mitochondrial particulate component in the castor bean endosperm. Since this particle also contains other enzymes of the glyoxylate cycle it seems probable that this is the site of the reactions *in vivo*. With the

exception of aconitase, which is unstable in these extracts, all of the enzymes needed for the net synthesis of succinate from acetyl CoA units are present in the purified glyoxysome fraction. The source of acetyl CoA in vivo is β-oxidation of the long-chain fatty acids (Yamada and Stumpf, 1965; Rebeiz and Castelfranco, 1964) and the fate of succinate is conversion to sucrose via phosphoenolpyruvate and EMP sequence (Beevers, 1961). The intracellular localization of these reaction sequences and the interplay between them and the glyoxysome requires investigation. The confining of the glyoxylate cycle reactions to a distinct organelle lacking several of the oxidative enzymes of the TCA cycle simplifies the problem of regulating the flow of carbon at the level of isocitrate (Tanner and Beevers, 1965) and also puts the question of development and decline of the enzymes during germination (Beevers, 1961) in a different light.

In previous work with Tetrahymena (Hogg and Kornberg, 1963) it was concluded that the functional enzymes of the glyoxylate cycle were confined to a special mitochondrial fraction. Very recently Müller and Hogg (1967) have shown that malate synthetase and isocitrate lyase are associated with a particle, the peroxisome, which however lacks several other enzymes of the glyoxylate cycle. In Chlorella cells with a functional cycle, isocitrate lyase was associated with a dense particulate fraction (Harrop and Kornberg, 1966). Thus there are precedents from work with other organisms that at least some of the enzymes of the glyoxylate cycle are specially localized.

Although a close comparison with these studies is not possible it is clear that the particles from castor bean endosperm which contain the unique enzymes of the glyoxylate cycle are not mitochondria; they sediment differently and they do not contain fumarase, NADH oxidase, succinate dehydrogenase or cytochromes. Since they do contain the enzymatic equipment for the conversion of acetate to succinate in the glyoxylate cycle we refer to them as glyoxysomes. Similar particles have recently been isolated from watermelon cotyledons in this laboratory. Further

characterization of these particles from both castor bean and watermelon storage tissues is in progress.

ACKNOWLEDGMENTS

This investigation was supported in part by a Public Health fellowship 1-F2-GM-23,105-01 from the National Institute of General Medical Sciences, and by NSF Grant GB-2599. We thank Dr. Albert Kahn and Mrs. Dorothy Werderitsh for their generous assistance with the electron and light microscopic phases of this study.

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