Subcellular Distribution of Gluconeogenic Enzymes in Germinating Castor Bean Endosperm

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ABSTRACT

The intracellular distribution of enzymes capable of catalyzing the reactions from oxaloacetate to sucrose in germinating castor bean endosperm has been studied by sucrose density gradient centrifugation. One set of glycolytic enzyme activities was detected in the plastids and another in the cytosol. The percentages of their activities in the plastids were less than 10% of total activities except for aldolase and fructose diphosphatase. The activities of several of the enzymes present in the plastids seem to be too low to account for the in vivo rate of gluconeogenesis so that those in the cytosol are quite adequate. Furthermore, phosphoenolpyruvate carboxylase, sucrose phosphate synthetase, and sucrose synthetase, which catalyze the first and final steps in the conversion of oxaloacetate to sucrose, were found only in the cytosol. It is deduced that in germinating castor bean endosperm the complete conversion of oxaloacetate to sucrose and CO₂ occurs in the cytosol. The plastids contain some enzymes of the pentose phosphate pathway, pyruvate dehydrogenase and fatty acid synthetase in addition to the set of glycolytic enzymes. This suggests that the role of the plastid in the endosperm of germinating castor bean is the production of fatty acids from sugar phosphates, as it is known to be in the endosperm during seed development.

There is a massive conversion from fat to sucrose in the endosperm of young castor bean seedlings. It has been demonstrated (see Fig. 7) that the oxidation of fatty acids and the subsequent conversion of acetyl-CoA to succinate occur in glyoxysomes and that succinate is converted to malate or oxaloacetate in the mitochondria (4). However, the intracellular site of the reactions from oxaloacetate to sucrose is still not known. Kobr and Bevers (18) have shown that the concentrations of glycolytic intermediates and coenzymes in castor bean endosperm do not differ strikingly from those in nongluconeogenic tissues such as pea and buckwheat seedlings. They suggested (17) that gluconeogenesis may occur in a separate intracellular compartment, the plastid, since several glycolytic enzyme activities were found there. In a previous paper we described a method for preparing intact plastids in high yield from protoplasts from castor bean endosperm (24) which allows a quantitative approach to this problem. We report here the intracellular localization of gluconeogenic and related enzymes using this method, and conclude that the production of sucrose from oxaloacetate occurs in the cytosol.

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MATERIALS AND METHODS

Seeds of castor bean (Ricinus communis cv. Hale) were soaked in running tap water for 1 day and germinated in moist Vermiculite at 30 C. The methods for preparation of protoplasts from the endosperm of 4-day-old castor bean seedlings and disruption of the protoplasts in 150 mM Tricine-KOH (pH 7.5)-25% sucrose-1 mM EDTA were described previously (24). The ruptured protoplasts (about 10 mg protein in 2 ml) were layered on a gradient composed of: (a) a 1-ml cushion of 60% (w/w) sucrose; (b) 13 ml sucrose solution increasing linearly from 30 to 60% sucrose; and (c) 1 ml 30% sucrose in an 18-ml tube. All sucrose solutions were prepared in 0.1 mM EDTA (pH 7.5). The gradients were centrifuged at 21,000 rpm for 3 h in a Beckman model L2-65B centrifuge in a Spincow SW 27-1 rotor at 4 C, and sequential fractions (0.4 ml) were collected.

Enzyme Assays. All assays except those for sucrose-P synthetase (37 C), sucrose synthetase (37 C), RuP₂ carboxylase (25 C), and fructose diphosphatase (25 C) were carried out at room temperature (about 23 C). Assays for sucrose-P synthetase and sucrose synthetase were carried out by measuring the incorporation of labeled UDP-glucose into sucrose-P or sucrose at 37 C for 30 min by a modification of Hawker's methods (14). The reaction mixtures contained, in µmol, K-phosphate buffer (pH 7.5), 5.0; EDTA, 2.5; NaF, 1.25; fructose-6-P, 2.5; UDP-[¹⁴C]glucose, 0.5 (1 µCi); in a total volume of 75 µl for sucrose synthetase. The reactions were stopped by heating in boiling water for 2 min. Twenty µl of reaction mixture were applied to a paper chromatogram. The radioactivity incorporated into sucrose was measured by cutting the chromatogram into 1-cm-wide strips which were assayed for ¹⁴C in scintillator solution (0.5%, w/v, PPO in toluene) after chromatography in ethyl acetate-pyridine-water (2:2:1, v/v/v) for 20 h. For the sucrose-P synthetase assay the remainder of the reaction mixture (50 µl) was added to 0.1 ml of 50 mM glycine-NaOH buffer (pH 10.6) containing MgCl₂ (5 µmol), 2 µl alkaline phosphatase (Sigma type III), and toluene (10 µl). After incubation at 30 C for 15 h, 20-µl samples were chromatographed as above. The differences between the ¹³C in sucrose before and after phosphatase treatment represent incorporation of ¹³C into sucrose-P by sucrose-P synthetase. It was proved that the product was sucrose by invertase treatment (data not shown). The pH of the assay mixture is the optimum pH determined for these enzyme activities in crude homogenates. Hexokinase, hexose-P isomerase, glucose-6-P dehydrogenase, 6-P-glucuronate dehydrogenase, phosphoglucomutase, UDP-glucose pyrophosphorylase, 3-P-glycerate kinase, P-glycerate mutase, pyruvate kinase, transaldolase, transketolase, phosphofructokinase, enolase, NAD and NADP triose-

4 Abbreviations: UDP-glucose: uridine diphosphate glucose; PEP: phosphoenolpyruvate; RuP₂: ribulose 1,5-bisphosphate.
P dehydrogenase were assayed according to Simcox et al. (32). Other assays were those described in the literature as follows, catalase (22), fumarase (28), RuP2 carboxylase (26), PEP carboxylase (8), PEP carboxykinase (8), pyruvate dehydrogenase (29), aldolase (15), fructose diphosphatase (7), NAD malate dehydrogenase (3), triose-P isomerase (23). Coupling enzymes were obtained from Sigma.

RESULTS

In a previous paper we reported methods for preparing intact plastids in high yield from protoplasts from castor bean endosperm. The enzyme profile of the broken protoplast preparations after sucrose density gradient centrifugations is shown in Figure 1. The prominent protein peak coincides with fumarase activity which is the marker enzyme of mitochondria. Less than 5% of the total fumarase activity was detected in the supernatant fractions. Catalase, the marker enzyme of glyoxysomes, is localized in a fraction at peak density 1.25 g/cm³ (see below). Only 7% of the total catalase activity was found in the supernatant fractions. RuP2 carboxylase, the marker enzyme of plastids, was clearly present in a fraction with peak density 1.22 g/cm³ and less than 2% of the total RuP2 carboxylase activity was detected in the supernatant fractions. These results show that these three organelles are recovered intact in high yield by this method (24). The distribution of the enzymes which are concerned with gluconeogenesis and related processes was examined in such gradients.

Localization of Enzymes Concerned with the Sequence from Oxaloacetate to Sucrose. It has been demonstrated that PEP carboxykinase which catalyzes the reaction from oxaloacetate to PEP and CO₂ (Fig. 7) plays a role in gluconeogenesis in germinating castor bean endosperm (5). This activity is detected only in the supernatant fractions (cytosol) which support previous conclusions (8, 20); no activity is present in the plastid fraction (Fig. 2b). The glycolytic enzymes enolase (Fig. 5b), P-glycerate mutase (Fig. 2a), 3-P-glycerate kinase (Fig. 6c), aldolase (Fig. 4a), fructose diphosphatase (Fig. 4a), hexose-P isomerase (Fig. 3b), phosphoglucomutase (Fig. 3b) as well as phosphofructokinase (Fig. 5b) and pyruvate kinase (Fig. 6b) are clearly present in the plastids as well as in the supernatant fractions. Relatively high amounts of aldolase and fructose diphosphatase are detected in plastid fractions. UDP-Glucose pyrophosphorylase is found in the plastid fractions but there is no peak (Fig. 6c). Sucrose-P synthetase and sucrose synthetase, concerned with sucrose synthesis and breakdown (14), are detected in the supernatant fractions and are not present in the plastid fractions (Fig. 1e). These results show that the cells contain two sets of glycolytic enzymes, one in the plastids and one in the cytosol, but the enzymes catalyzing the first and final steps from oxaloacetate to sucrose are found only in the cytosol.

Localization of Other Related Enzymes. Some enzymes of the pentose cycle, 6-P-glucuronate dehydrogenase (Fig. 3a), transaldolase (Fig. 3a), and transketolase (Fig. 5a) are also found in the plastid fractions as reported for the developing castor bean endosperm (32). Most of the pyruvate dehydrogenase activity (Fig. 2a) is found in the mitochondria; the plastid fractions show a significant but small activity and none is detected in supernatant fractions. Glucose-6-P dehydrogenase (Fig. 3a) and NADP-triose-P dehydrogenase (Fig. 4b) known to be present in chloroplasts (25, 30) are not detected in the plastid fractions. NAD malate dehydrogenase (Fig. 6a) activity is found in mitochondrial fractions, glyoxysomal fractions, and supernatant fractions as shown earlier (16) but no clear peak is present in the plastids. Particulate hexokinase activity (Fig. 6b) coincides with the mitochondrial fractions. PEP carboxylase activity (Fig. 2b) is found only in the supernatant fractions as reported in the mesophyll cells of C₃ plants (13). In Tables I and II the contributions of plastid and cytosol to the total enzyme activities are shown; fractions 1 to 8 from the gradient are taken to represent the cytosol activity while fractions 20 to 28 represent the plastids.

DISCUSSION

The isolation of plastids in essentially complete yield from protoplasts has allowed a direct assessment of the amounts of individual enzymes present in these organelles. One difference between the results in this paper and those reported previously (24), which has no bearing on the conclusions, should be noted. In the earlier report, in which castor beans from the 1976 harvest were used for protoplast and organelle preparation, the glyoxysomes were (and are) retained at a density of 1.13 g/cm³ in the gradients whereas in the conventional preparations from the same endosperm tissue the glyoxysomes appear at their known equilibrium density of 1.25 g/cm³ (24). In all of the experiments reported here freshly harvested beans from 1977 were used and from these the glyoxysomes obtained from protoplasts or from grinding the endosperm appear in the gradients at a mean density of 1.25 g/
Fig. 2. Distribution of: (a) P-glycerate mutase, pyruvate dehydrogenase, (b) PEP carboxylase, PEP carboxykinase in a sucrose gradient. In this and subsequent figures, arrows show peaks of fumarase and catalase activities in gradient, and one enzyme unit is 1 nmol substrate consumed/min.

Fig. 3. Distribution of: (a) glucose-6-P dehydrogenase, 6-P-gluconate dehydrogenase, (b) hexose-P isomerase, phosphoglucomutase in a sucrose gradient.

We have no explanation for this consistent difference in behavior of the glyoxysomes; the yield of intact plastids and their sedimentation in the sucrose gradient are the same from all of the protoplast preparations that we have used.

In considering the plastid as a possible site of reversed glycolysis (as it is in photosynthesis) the results in Table I show that except for aldolase and fructose diphosphatase, the activities of the glycolytic enzymes in the plastids are all less than 10% of the total (Table I) and some, e.g. enolase and P-glycerate mutase, are less than 1%.
To account for the maximum in vivo rate of sucrose synthesis, 114 nmol sucrose/min-endosperm at 25 C (9), each enzyme present in the tissue must be capable of handling its substrate at a corresponding rate. The total amount of protein in the endosperm at 4 days is 30 mg. Since the amount of total protein from the protoplasts applied to the gradients was about 10 mg, a minimum capacity of each enzyme corresponding to a rate of sucrose synthesis of 38 nmol/min in the total plastid fraction would be required. It is clear that the cytosolic components of the enzymes are adequate or greatly exceed this requirement (Table I). Within the plastids only the activities of 3-P-glycerate kinase, triose-P-isomerase and aldolase exceed the requirement and those of enolase, P-glycerate mutase, and triose-P dehydrogenase are far below the minimum. On the assumption that the activities measured in in vitro assays after gradient separation reflect in vivo capacities, we concluded that the maximum flux of carbon through this pathway in the plastids is quite inadequate to account for the rate of sucrose synthesis observed. Thus, reversed glycolysis must occur in the cytosol (Fig. 7). A similar conclusion, based on less extensive data, was reached from work with marrow seedlings (2).

The production of PEP is exclusively cytosolic, and, like chloroplasts (6) the plastids cannot synthesize sucrose from its immediate precursors (Table I). The activity of sucrose synthetase in the cytosol exceeds that of sucrose-P synthetase, as reported

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**Fig. 4.** Distribution of: (a) aldolase, fructose diphosphatase, (b) NAD-triose-P dehydrogenase, NADP-triose-P dehydrogenase in a sucrose gradient.

**Fig. 5.** Distribution of: (a) transaldolase, transketolase, (b) phosphofructokinase, enolase in a sucrose gradient.
Fig. 6. Distribution of: (a) NAD malate dehydrogenase, triose-P isomerase, (b) pyruvate kinase, hexokinase, (c) UDP-glucose pyrophosphorylase, 3-P-glycerate kinase in a sucrose gradient.

Fig. 7. Intracellular distribution of enzyme systems responsible for conversion of fatty acid to sucrose in castor bean endosperm. In this representation malate leaves the mitochondria and is oxidized in the cytosol, yielding the NADH required for reduction of 1,3-diphosphoglycerate to triose-P. The vacuole is shown as a repository of sucrose (23).
Values in parentheses show percentages of total activity in the gradient.

<table>
<thead>
<tr>
<th>CYTOSOL</th>
<th>PLASTIDS</th>
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<td>nmoles substrate used/min</td>
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### Table I. Gluconeogenic Enzymes in Plastids and Cytosol.

The observed levels of sucrose-P synthetase (Table I) are barely adequate for in vivo sucrose synthesis but these are minimal values. Although the enzyme is activated by Fe²⁺ and K⁺ (31) these ions were deliberately omitted from our assays because they also may increase the breakdown of sucrose-P by the phosphatase.

Since the plastids do not appear to be the site of reversed glycolysis, the question of the function of the glycolytic pathway in these organelles arises. In the endosperm tissue during early growth, fatty acids destined for incorporation into phospholipids are synthesized (10); this synthesis occurs in the plastids (33). The fact that the plastids have the capacity to produce acetyl-CoA from sugar phosphates, as well as the NADH and NADPH required for fatty acid synthesis, suggests that these organelles may be the site of the over-all reactions from sugar phosphates to fatty acids as they are known to be in the ripening seed (32, 34).

The plastid ATP-citrate lyase recently described (11) offers an additional source of acetyl-CoA for fatty acid synthesis.

In leaves, chloroplasts contain their own set of glycolytic enzymes (e.g. 1, 19, 30) including glucose-6-P dehydrogenase and NADP triose-P dehydrogenase (25, 30). Neither of these latter two enzymes is present in the plastids of the endosperm from germinating castor bean, and the glucose-6-P dehydrogenase is not present in the plastids from endosperm in the ripening seedling (32). As in the chloroplasts, several of the plastid enzymes in ripening seeds have been shown to be isoenzymes separable from their cytosolic counterparts (21, 31) and an investigation of the isoenzymes in the germinating castor bean is now proceeding. The recognition that even in nongreen cells the plastids house glycolytic enzymes means that these can no longer be regarded as unique cytosolic markers. It appears justifiable to continue to retain sucrose-P synthetase, sucrose synthetase, PEP carboxylase and PEP carboxykinase in this category.

### Table II. Other Enzymes in Plastids and Cytosol.

Values in parentheses show percentages of total activity in the gradient.

<table>
<thead>
<tr>
<th>CYTOSOL</th>
<th>PLASTIDS</th>
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<tr>
<td>nmoles substrate used/min</td>
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—No clear peak in plastids.

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