Enzymes of Glycerol Metabolism in the Storage Tissues of Fatty Seedlings

Received for publication September 18, 1974 and in revised form November 27, 1974

ANTHONY H. C. HUANG
Department of Biology, University of South Carolina, Columbia, South Carolina 29208

ABSTRACT

Various enzymes of glycerol metabolism in the extracts of 5-day-old castor bean (Ricinus communis L. var. Hale) endosperm and 4-day-old peanut (Arachis hypogaea L.) cotyledon were studied. NAD-glycerol dehydrogenase and NAD-\(\alpha\)-glycerolphosphate dehydrogenase were not detected. Glycerol kinase was detected in the soluble fractions and an \(\alpha\)-glycerolphosphate oxidoreductase was found in the particulate fractions. The particulate fractions were separated into various organelle fractions by sucrose gradient centrifugation and the \(\alpha\)-glycerolphosphate oxidoreductase was shown to be present in the mitochondria. The properties of the castor bean mitochondrial \(\alpha\)-glycerolphosphate oxidoreductase resembled those of a similar enzyme present in the mitochondria of many animal tissues. A survey showed that the \(\alpha\)-glycerolphosphate oxidoreductase was present in great amount only in the storage tissues of fatty seedlings but not in other nonfatty plant tissues. It is concluded that in the storage tissues of fatty seedlings, the soluble glycerol kinase and the mitochondrial cytochrome-linked \(\alpha\)-glycerolphosphate oxidoreductase are the two enzymes responsible for the initial conversion of glycerol to hexose.

During germination of fatty seeds, the reserve lipid is converted efficiently to sucrose. This gluconeogenesis pathway and the subcellular localization of the enzymes involved have been studied intensively. Triglycerides are first hydrolyzed by an acid lipase in the spherosomes (15) and an alkaline lipase in the glyoxysomes (14). The fatty acids are then oxidized by the enzymes of the \(\beta\)-oxidation and of the glyoxylate cycle inside the glyoxysomes (3, 10). The product succinate is further metabolized by the enzymes of the tricarboxylic acid cycle in the mitochondria and by the enzymes of the Embden-Meyerhof-Parnas pathway in the cytosol to form hexose (3).

Most of the glycerol released after the hydrolysis of the glycerides is converted to sucrose as shown by labeling experiments with radioactive glycerol in castor bean endosperm (1; H. Bevers, personal communication). The glycerol is presumably first converted by two enzymatic reactions to dihydroxyacetone phosphate which will be further metabolized to hexose by the reversal of the Embden-Meyerhof-Parnas pathway (2). The enzymes involved for the conversion of glycerol to dihydroxyacetone-P and their subcellular localization are less understood. Stumpf (18) reported that in peanut cotyledon a cytochrome c-linked glycerol-P dehydrogenase was present in the particulate organelle fraction. This particulate fraction has been shown recently to contain mitochondria, glyoxysomes, and endoplasmic reticulum (13), and thus the subcellular localization of the enzyme is still uncertain. The subcellular localization of the glycerol kinase was not examined but was suggested to be in the mitochondria (18). In contrast, working with the endosperm of germinating castor bean, Yamada (20) failed to detect the particulate glycerol-P dehydrogenase and found that glycerol kinase and a soluble NAD-linked glycerol-P dehydrogenase were both present in the soluble fraction.

In this paper, the enzymes of glycerol metabolism and their subcellular localization in different fatty seedlings are reported. The data suggest that in peanut cotyledons and in castor bean endosperm, a cytosol glycerol kinase and a mitochondrial Cytochrome-linked glycerol-P oxidoreductase are the two enzymes responsible for the initial steps of glycerol metabolism.

MATERIALS AND METHODS

Plant Materials. Seeds were soaked in running tap water for 1 day and then allowed to germinate in moist vermiculite at 30 C in darkness. Endosperm and roots of 5-day-old castor bean (Ricinus communis L. var. Hale), cotyledons of 4-day-old watermelon seeds (Citrullus vulgaris Schrad.), peanut seeds (Arachis hypogaea L.), cucumber seeds (Cucumis sativus L.) and pea seeds (Pisum sativum L.), megagametophyte of 9-day-old pine seeds (Pinus ponderosa L. var. Colorado), potato tubers (Solanum tuberosum L.), spinach leaves (Spinacia oleracea L.), cauliflower bud (Brassica oleracea L.), and rat liver were used. The tissues were first chopped to small pieces with razor blades in grinding medium and then ground gently with a mortar and pestle (7). The grinding medium was that described previously (7) except that 2 mM dithiothreitol was used. After centrifugation at 270g for 10 min to remove cell debris, the homogenate was centrifuged at 10,000g for 30 min to yield a supernatant fraction and a particulate fraction (7). The preparation of organelles in sucrose gradient from castor bean endosperm and peanut cotyledon followed that described previously (7, 8). The supernatant fractions obtained after centrifuging the crude homogenate for 10 min at 270g were layered directly onto the gradients. Beckman J21B centrifuge and Beckman L2-65B ultracentrifuge with Rotor SW27 were used for centrifugation.

Enzyme Assays. A Beckman Acta II double beam spectrophotometer attached to a Beckman 1005 recorder was used to monitor changes in absorbance. Oxygen electrode Model 53 from Yellow Springs Instrument Co. was used to measure \(\text{O}_2\) uptake. NAD-glycerol-P dehydrogenase was assayed by a modified method (5) in 0.05 M triethanolamine-HCl buffer, pH 7.5, containing 0.14 mM NADH, and 0.2 mM dihydroxyacetone-P.
### Table I. Activities of Various Enzymes in Castor Bean Endosperm and Peanut Cotyledon

<table>
<thead>
<tr>
<th></th>
<th>Castor Bean Endosperm</th>
<th>Peanut Cotyledon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total activity (nmol/min·mg fresh wt)</td>
<td>Distribution (10,000g supernatant/Pellet)</td>
</tr>
<tr>
<td>Catalase</td>
<td>14,900,000</td>
<td>25/75</td>
</tr>
<tr>
<td>Fumarase</td>
<td>1,840</td>
<td>10/90</td>
</tr>
<tr>
<td>Glycerol kinase</td>
<td>23</td>
<td>100/0</td>
</tr>
<tr>
<td>α-Glycerol-P oxido-</td>
<td>340</td>
<td>6/94</td>
</tr>
<tr>
<td>reductase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose 1,6-di-P</td>
<td>1,030</td>
<td>91/9</td>
</tr>
<tr>
<td>aldolase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triose-P isomerase</td>
<td>438,000</td>
<td>95/5</td>
</tr>
<tr>
<td>Fructose 1,6-di-P</td>
<td>1,120</td>
<td>99/1</td>
</tr>
</tbody>
</table>

Glycerol-P dehydrogenase was assayed at pH 9 in bicarbonate buffer as described earlier (12). α-Glycerol-P oxidoreductase was measured by a modified method (17) in 0.05 M potassium phosphate buffer, pH 7.5, containing 1 mm KCN, 0.05% phenazine methosulfate, 0.05 mm 2,6-dichlorophenol-indophenol, and 0.2 M D,L-α-glycerol-P. The assays of catalase, fumarase (7), and fructose 1,6-diphosphatase (9) followed those described previously. Triose-P isomerase was assayed according to Beisenherz (4) using 0.1 M triethanolamine buffer, pH 7.6. Fructose 1,6-di-P aldolase was assayed by coupling the reaction with triose-P isomerase and NAD-glycerol-P dehydrogenase (6). Glycerol kinase was assayed by phosphorilation of added glycerol from radioactive (γ-32P)-ATP into acid-stable phosphorylated compounds (19). The reaction mixture contained in a volume of 1 ml, 0.1 M tris-HCl buffer, pH 8.5, 1 mm ATP, 1 mm MgCl2, 1 mm glycerol, and enzyme preparation. (γ-32P)-ATP was obtained from New England Nuclear Co. and approximately 200,000 cpm/μmole were used. The radioactivity was measured as described (19) in a Perkin Tri-Carb scintillation counter.

When the supernatant and the particulate fractions were assayed for glycerol kinase, only the supernatant fraction showed substantial activity whereas the particulate fraction was completely devoid of the enzyme activity. However, the activity in the supernatant fraction was quite independent of added glycerol, presumably because of the presence of glycerol in that fraction. Also, a comparatively active glucose kinase (unpublished) together with a fairly high glucose content (9) in the supernatant fraction would mask the glycerol kinase assay system. To resolve these problems, the small molecules in the supernatant fraction were removed by the following treatment. The fraction was made 80% (56.1 g/100 ml) with ammonium sulfate, and the precipitate was resuspended in 0.1 M tris-HCl buffer, pH 8.5, and was desalted by passing through a column of Sephadex G-25 equilibrated with the same buffer. The void volume was collected and assayed for glycerol kinase activity again. The preparation showed enzyme activity that was dependent on added glycerol, and the values are shown in Table I.

### RESULTS AND DISCUSSION

**Enzymes of Glycerol Metabolism.** Endosperm of 5-day-old castor bean and cotyledons of 4-day-old peanut were ground in a grinding medium carefully and the extracts were centrifuged at 10,000g to give a supernatant fraction and a particulate fraction. The activities of various enzymes were assayed in these two fractions. As shown in Table I, substantial activities of fumarase (marker enzyme of mitochondria) and catalase (marker enzyme of glyoxysomes) were recovered in the particulate fraction, indicating that the various organelles were well preserved after grinding. NAD-glycerol dehydrogenase and NAD-α-glycerol-P dehydrogenase were not detected. Glycerol kinase was detected in the extracts of these two tissues and all of the activity was present in the soluble fractions (Table I). The activity in the extract of peanut cotyledon is high enough to account for the in vivo rate of gluconeogenesis whereas that in the castor bean endosperm is only slightly lower than the necessary amount. The latter activity is already much higher than that reported earlier in the same tissue (20). Using phenazine methosulfate and 2,6-dichlorophenol-indophenol as electron acceptors, an α-glycerol-P oxidoreductase was detected mainly in the particulate fractions of the two tissues and the activities are more than enough to account for the in vivo rate of gluconeogenesis. Most of the activities of fructose 1,6-di-P aldolase, triose-P isomerase, and fructose 1,6-diphosphatase, the initial three enzymes in the Embden-Meyerhof-Parnas pathway responsible for the conversion of dihydroxyacetone phosphate to glucose-P were present in the soluble fractions.

The presence of α-glycerol-P oxidoreductase in the particulate fractions of the two tissues agrees with that observed in peanut cotyledon (18) and negates the report that such an enzyme was absent in castor bean endosperm (20). The particulate fraction from fatty seedlings has been shown recently (13) to contain many different organelles including mitochondria, glyoxysomes, and endoplasmic reticulum, and thus the subcellular localization of the enzyme is still uncertain. To elucidate this point, the various organelles in the extracts of castor bean endosperm and peanut cotyledons were separated by sucrose gradient centrifugation and their distribution across the gradients is shown in Figure 1. As described in previous reports (13), Cyt c reductase showed a major peak at 1.12 g/cm³ and a minor peak at 1.18 g/cm³, corresponding to the endoplasmic reticulum and mitochondria, respectively. The mitochondrial peak at 1.18 g/cm³ was also demonstrated by the distribution of fumarase, a marker enzyme of the organelles. Isocitrate lyase, a marker enzyme of glyoxysomes, peaked at 1.25 g/cm³. α-Glycerol-P oxidoreductase followed a similar distribution as that of fumarase and thus it is localized in the mitochondria rather than the endoplasmic reticulum or the glyoxysomes.

**Properties of Mitochondrial α-Glycerol-P Oxidoreductase.** The mitochondrial fraction obtained from sucrose gradient prepared from castor bean endosperm was used to study the properties of α-glycerol-P oxidoreductase. Using phenazine methosulfate and 2,6-dichlorophenol-indophenol as electron acceptors, the enzyme has a pH optimum for activity around pH 6 to 7.5, and the apparent Km value for α-α-glycerol-P is 50 mm. This relatively high apparent Km value was also reported earlier for the peanut enzyme (18). The activity is inhibited by various detergents but not by 2 M KCl or 2% ethanol. The inhibition is 94% with 0.2% Triton X-100, 68% with 0.5% Tween 20, and 71% with 0.5% digitonin. The preparation can reduce Cyt c in the presence of potassium cyanide (5 mm) and α-glycerol-P and this reduction is completely inhibited by antimycin A (20 pmoles/mg protein) or 1% ethanol. The preparation can also take up O₂ in the presence of α-glycerol-P and the activity is completely abolished by 5 mm potassium cyanide or antimycin A (20 pmoles/mg protein). The enzyme
activity is preserved after the preparation has been frozen for several months. The localization in the mitochondria, the relatively high Km value for α-glycerol-P, the possible membrane
association, and the linkage to Cyt all indicate that the properties of the castor bean mitochondrial enzyme resemble those of a similar enzyme described in the mitochondria of many ani-
mal tissues (11, 16, 17). Whether or not the castor bean en-
zyme is linked to Cyt b requires further investigation.

α-Glycerol-P Oxidoreductase in Various Plant Tissues. In
animal tissues the mitochondrial α-glycerol-P oxidoreductase and the soluble NAD-α-glycerol-P dehydrogenase are believed to function as an electron shuttle system in the oxidation of extramitochondrial NADH (11). A survey of the mitochondrial α-glycerol-P oxidoreductase in various plant tissues (Table II) shows that the enzyme is present in great amounts only in the storage tissues of farty seedlings but not in a variety of other
nonfatty plant tissues. NAD-α-glycerol-P dehydrogenase is ab-
sent in all plant tissues tested except potato tuber which con-
tains a very low activity of the enzyme. In rat liver, the activity
of NAD-α-glycerol-P dehydrogenase is very high and more than 95% of it was recovered in the soluble fraction. The activity
of α-glycerol-P oxidoreductase in rat liver is comparable to
those in the storage tissues of fatty seedlings and most of it is
present in the particulate fraction. The data thus strongly
suggest that the α-glycerol-P oxidoreductase in fatty seedlings
serves the purpose of glycerol metabolism rather than electron
shuttle as suggested in many animal systems.

From the data presented, it is concluded that in the storage
tissues of fatty seedlings, the initial step for the conversion of
glycerol derived from stored lipid to hexose involves two enz-
zymatic reactions. The glycerol released after the hydrolysis of
glycerides by the acid and alkaline lipases (14, 15) is phos-
phorylated by a glycerol kinase in the cytosol to give α-glyc-
rol-P which is in turn oxidized by a mitochondrial cytochrome
linked α-glycerol-P oxidoreductase. The product, dihydroxyacetone-P,
is released to the cytosol and converted to hexose by the en-
zymes of the Embden-Meyerhof-Parnas pathway.

Acknowledgments—I wish to thank Dr. Harry Beevers and Mr. John
Sorensen for their valuable suggestions.

Table II. Activities of α-Glycerolphosphate Oxidoreductase and
NAD-α-glycerol-P Dehydrogenase in Various Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>α-Glycerol-P Oxidoreductase</th>
<th>NAD-α-glycerol-P Dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castor bean endosperm</td>
<td>340</td>
<td>0</td>
</tr>
<tr>
<td>Peanut cotyledon</td>
<td>250</td>
<td>0</td>
</tr>
<tr>
<td>Cucumber cotyledon</td>
<td>320</td>
<td>0</td>
</tr>
<tr>
<td>Watermelon cotyledon</td>
<td>215</td>
<td>0</td>
</tr>
<tr>
<td>Pine megagametophyte</td>
<td>264</td>
<td>0</td>
</tr>
<tr>
<td>Potato tuber</td>
<td>0.7</td>
<td>3.4</td>
</tr>
<tr>
<td>Spinach leaves</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pea cotyledon</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cauliflower bud</td>
<td>8.2</td>
<td>0</td>
</tr>
<tr>
<td>Castor bean root</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rat liver</td>
<td>350</td>
<td>28,000</td>
</tr>
</tbody>
</table>

Fig. 1. Separation of organelles from 5-day-old castor bean
endosperm and 4-day-old peanut cotyledon on sucrose gradients.
The supernatant fractions obtained after centrifugation of the crude
extract for 10 min at 270g were layered onto the gradient. The
dashed lines mark the peaks of endoplasmic reticulum (density 1.12),
mitochondria (density 1.19), and glyoxysomes (density 1.25). Units
per gradient fraction: A: 280 nm; relative; all enzymes: µmoles/min.