ATP Citrate Lyase from Germinating Castor Bean Endosperm

LOCALIZATION AND SOME PROPERTIES

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ABSTRACT

ATP citrate lyase (EC 4.1.3.8) has been found in crude extracts from endosperm tissue of germinating castor bean and shows its maximum activity in 4- to 5-day-old seedlings. A strict requirement for coenzyme A and adenosine 5'-triphosphate was demonstrated. The pH optimum for the reaction is around 7.5. The unstable enzyme can be stabilized by freezing and addition of citrate and glycerol. (−)-Hydroxycitrate is a potent inhibitor. The molecular weight is about 400,000. The adenosine 5'-triphosphate citrate lyase is localized in the plastids, where it possibly plays a role in providing acetyl coenzyme A for lipid biosynthesis.

The ATP citrate lyase (ATP: citrate oxaloacetate lyase, CoAacylating, EC 4.1.3.8. ACL, and also called citrate cleavage enzyme) catalyzes the following reaction:

\[
\text{citrate} + \text{ATP} + \text{CoA} \rightarrow \text{oxaloacetate} + \text{acetyl-CoA} + \text{ADP} + \text{Pi}
\]

This enzyme was first described by Serer and Lipmann (18) in pigeon liver and has been further characterized in chicken and rat liver (17). It is widely distributed in animal tissues and shows high activity in liver, adipose tissue, lactating mammary glands, brain and adrenal tissue (7, 16, 17). ACL was found to be localized in the cytosol (16). (−)-Hydroxycitrate has been shown to be a potent and specific competitive inhibitor of the enzyme (17). In animals, particularly those on a high carbohydrate diet, ACL plays an important role in fatty acid synthesis (17). Citrate generated in the mitochondria is cleaved by ACL in the cytosol, providing the acetyl-CoA used there in fatty acid biosynthesis. Accordingly, exogenous citrate is a good precursor of fatty acids and (−)-hydroxycitrate is an inhibitor of fatty acid synthesis from suitable precursors in vivo (17).

The ACL is clearly different from the bacterial citrate lyase which cleaves citrate to oxaloacetate and acetate in the absence of ATP and CoA. The ATP requirement for the ACL reaction distinguishes this reaction from a reversal of the citrate synthase reaction in which citrate is formed from acetyl-CoA and oxaloacetate. Little is known about the occurrence and significance of ACL in higher plants. Mattow and Modl (6) showed it to be present in ripening mango fruit and Nelson and Rinne (8) have reported its occurrence in developing soybean cotyledons. Both groups suggest that ACL is involved in the production of extra-mitochondrial acetyl-CoA in higher plants.

MATERIALS AND METHODS

Castor bean seeds (Ricinus communis cv. Hale) were soaked overnight in running tap water, sown in water-saturated Vermiculite, and grown at 30 C in darkness. Endosperms were removed from seedlings, rinsed in ice cold water, and kept on ice until homogenization.

Crude extracts were prepared by grinding the endosperms from five seedlings in 5 ml 0.1 M Tricine-KOH (pH 7.5) containing 10 mM mercaptoethanol and 0.1% Triton X-100 in a chilled mortar. The resulting slurry was centrifuged at 30,000g for 20 min in a refrigerated centrifuge. The fat layer and pellet were discarded and the supernatant solutions used as the crude extract. Extracts of endosperm from ripening castor bean at the stage when the testae were just beginning to color were prepared in the same way. Seeds of watermelon (Citrullus vulgaris), sweet corn (Zea mays), soybean (Glycine max), sunflower (Helianthus annuus), peanut (Arachis hypogea), and mung bean (Phaseolus aureus) were germinated for castor beans. For the preparation of crude extracts, the roots and lower half of the hypocotyl were removed and the rest of the seedling homogenized and centrifuged as described above. From sweet corn the coleoptile (including the primary leaf) and scutellum were extracted. In preparing castor bean extract for the time course studies some sand was added to facilitate grinding.

Total citrate in the endosperm tissue was extracted in dilute HClO4 and estimated using the malate dehydrogenase coupled assay with commercial bacterial citrate lyase (2). Protein concentrations were determined by the biuret reaction or a modified Lowry procedure (3).

For the preparation of organelles, 20 isolated endosperms were chopped with razor blades in 14 ml 0.15 M Tricine-KOH (pH 7.5) containing 10 mM 2-mercaptoethanol, 1 mM EDTA, and 20% sucrose (w/w). The homogenate was passed through three layers of nylon cloth and 10 ml of the filtrate was layered on top of a linear sucrose gradient. The gradient consisted of 40 ml 30 to 60% sucrose containing 10 mM citrate and 1 mM EDTA, over a cushion of 62% sucrose, and topped by 4 ml 30% sucrose containing the same additives.

The gradients were centrifuged for 3.5 h at 21,000 rpm (Beckman rotor SW 25.2). The fat layer was removed and the gradient collected in 1.2-ml fractions. The sucrose concentration in the samples was determined refractometrically.

Gel filtration of crude extracts was performed on Bio-Gel A-5m (Bio-Rad). The column (55 x 2.5 cm) was equilibrated with the eluting buffer (0.1 M Tricine-KOH [pH 7.5] containing 10% glycerol, 10 mM citrate, 1 mM MgCl2, and 0.2 mM DTT). Five ml crude extract were applied to the column and 5-ml fractions were collected. For molecular weight estimations, the column was calibrated with 5 mg each of ferritin, catalase, and aldolase (Boehringer).

Enzyme activities in gradient samples were assayed according to published procedures as follows: catalase (5), aldolase (4), fumarase (13), malate dehydrogenase (11), citrate synthase (1), RuBP carboxylase (21), and pyruvate dehydrogenase complex.

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4 Abbreviations: ACL: ATP citrate lyase; RuBP: ribulose bisphosphate.
ATP citrate lyase was usually assayed by a slight modification of the coupled spectrophotometric method of Takeda et al. (20). The assay mixture contained, in a volume of 1 ml, 0.1 m Tricine-KOH (pH 7.5), 1 mM DTT, varying amounts of citrate, 2 mM ATP, 4 mM MgCl₂, 200 μM NADH, 12 units of added malate dehydrogenase, and 20 to 400 μl of enzyme sample. The A at 340 nm was monitored for a 5- to 10-min preincubation period. Then 40 μM CoA were added and the reaction followed for a further 5 to 10 min. The hydroxamate test and the radioactive assay with [1,5-¹⁴C]citrate were performed according to Takeda et al. (20).

RESULTS

The spectrophotometric assay (16, 20) that we have used is shown in Figure 1, carried out with a crude extract from endosperm tissue of 4-day-old castor bean seedlings. ATP, CoA-dependent production of oxaloacetate from citrate is monitored by the coupled oxidation of NADH in the presence of malate dehydrogenase. Endogenous NADH oxidation is not increased by ATP or CoA added alone. As shown in Figure 1 and summarized in Table 1 the reaction requires the addition of ATP and CoA.

However, the extracts themselves contain the substrate citrate (see below) and no increase in the rate of reaction is observed when more citrate is added (Table I). Removal of endogenous substrate from the extract by filtration on Sephadex G-25 or by overnight dialysis resulted in complete and irreversible inactivation of the ACL. When gel filtration was carried out in the presence of 10 mM citrate, 80% of the added ACL activity was recovered. Table I also shows that (-)-hydroxycitrate, a potent inhibitor of the animal ACL (17), inhibits the castor bean enzyme. Addition of mercaptoethanol or DTT to the extraction medium increased the activity of ACL in crude extracts and the further addition of Triton X-100 gave maximal activity (Table I). Under the assay conditions used, the reaction rate remained constant for at least 10 min and was proportional to protein concentration between 10 and 500 μg. The pH optimum for the ACL reaction was 7.5 (Fig. 2).

In additional experiments (not shown) the production of [¹⁴C]oxaloacetate was demonstrated from [1,5-¹⁴C]citrate and CoA-ester formation was demonstrated by the hydroxamate assay (20).

Developmental Changes. ACL activity cannot be detected in the extracts from dry seeds. Low activity is present at day 2; it rises to a peak at day 5 and declines sharply thereafter (Fig. 3B). The general pattern is thus similar to that of catalase and fumarase, the marker enzymes for glyoxysomes and mitochondria, the organelles principally involved in the conversion of fat to sucrose. The changes in the fresh weight of the endosperm during the same

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Table I  Properties of ATP citrate lyase from castor bean endosperm.

<table>
<thead>
<tr>
<th>Incubation or treatment</th>
<th>Activity (IU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>100</td>
</tr>
<tr>
<td>Crude extract, with additional citrate</td>
<td>100</td>
</tr>
<tr>
<td>Crude extract, minus ATP</td>
<td>0</td>
</tr>
<tr>
<td>Crude extract, minus CoA</td>
<td>0</td>
</tr>
<tr>
<td>Crude extract, plus (-)-hydroxycitrate, 200 μM</td>
<td>90</td>
</tr>
<tr>
<td>Crude extract, plus (-)-hydroxycitrate, 800 μM</td>
<td>55</td>
</tr>
<tr>
<td>After Sephadex G-25 treatment, without citrate</td>
<td>100</td>
</tr>
<tr>
<td>After Sephadex G-25 treatment, with citrate</td>
<td>0</td>
</tr>
<tr>
<td>Enzyme extraction without additions</td>
<td>43</td>
</tr>
<tr>
<td>Enzyme extraction with 1 mM dithiothreitol</td>
<td>50</td>
</tr>
<tr>
<td>Enzyme extraction with 10 mM mercaptoethanol</td>
<td>100</td>
</tr>
<tr>
<td>Enzyme extraction with 10 mM mercaptoethanol, plus 0.1% Triton X-100</td>
<td>200</td>
</tr>
</tbody>
</table>

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period and the total citrate content of the tissue are shown in Figure 3A. Substantial amounts of citrate are present in the dry seed and only minor changes are apparent until day 5 of development.

**Stability and Partial Purification of ACL.** In the crude extract ACL from castor bean is highly unstable. Storage at −20 C and addition of glycerol increased the stability significantly (Table II). Ethylene glycol was less effective than glycerol. When it was necessary to store the enzyme for longer than 1 day, citrate and glycerol were added to the crude extract before freezing.

It has not been possible so far to purify the ACL from crude extracts by fractionation with ammonium sulfate or acetone. Both procedures resulted in an almost complete loss of activity, even in the presence of citrate and glycerol.

However, some purification of the ACL has been achieved by gel filtration on agarose gel (Bio-Gel A-5m) (Fig. 4). The column buffer contained glycerol, citrate, and magnesium ions. Eighty-five per cent of the initial activity was lost, resulting in a purification factor of only 1.3. The ACL was eluted in two separate peaks. The first, with higher mol wt and a slightly higher total activity is referred to as ACL I and the other, ACL II. A complete separation of ACL I from citrate synthase and catalase was obtained. Chromatography on Sepharose 2B and on DEAE-Sephadex was not successful.

The behavior of castor bean ACL on agarose gel suggests a high mol wt. A comparison of the elution volume of ACL I with the elution volumes of known proteins (aldolase, catalase, and ferritin) allows an estimate of the mol wt of about 400,000 (Fig. 5). From the elution volume of ACL II its mol wt is roughly 100,000.

**Intracellular Localization.** For the intracellular localization of the ACL a whole homogenate was fractionated on a sucrose density gradient containing citrate. Figure 6 shows the distribution of the ACL and the marker enzymes for mitochondria (fumarase), plastids (RuBP carboxylase), and glyoxysomes (catalase) in the gradient. Although glyoxysomes and plastids are not separated completely by this method, the coincidence of ACL with the

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**Fig. 3.** Changes in fresh weight (○), citrate content (△), and activities of catalase (■), fumarase (●), and ATP citrate lyase (A—A) of castor bean endosperm during germination. "0" designates the day of sowing (soaked seeds).

**Table II Effect of treatments on stability of ACL in crude extracts**

<table>
<thead>
<tr>
<th>Temperature of Storage</th>
<th>Addition</th>
<th>Activity (%) after 0</th>
<th>1 day</th>
<th>2 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>None</td>
<td>100</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>10% glycerol</td>
<td>100</td>
<td>79</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>10% ethylene glycol</td>
<td>100</td>
<td>53</td>
<td>-</td>
</tr>
<tr>
<td>-20°C</td>
<td>None</td>
<td>100</td>
<td>77</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>10% glycerol</td>
<td>100</td>
<td>85</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>10% ethylene glycol</td>
<td>100</td>
<td>72</td>
<td>51</td>
</tr>
</tbody>
</table>
plastid marker RuBP carboxylase indicates the localization of the ACL in the plastids. The recovery of about 75% of the total RuBP carboxylase at the top of the gradient shows that considerable plastid breakage had occurred. Correspondingly, 65% of the ACL activity was also recovered at the top of the gradient. In gradients without citrate, only insignificant amounts of active ACL were recovered.

ACL activity was also detected in crude extracts of ripening castor beans, and from 4-day-old seedlings of watermelon, sweet corn, soybean, and sunflower (Table III). No activity was detected in extracts from peanut and mung bean seedlings or in the green primary leaves of watermelon.

**DISCUSSION**

ATP citrate lyase activity has been demonstrated in crude extracts of endosperm from young castor bean seedlings; the formation of oxaloacetate was strictly dependent on ATP and CoA. Thus the reaction may be different from the system studied by Nelson and Rinne (8, 9) in the developing soybean seed. The substrate, citrate, is present in the extracts, and its removal by gel filtration or during conventional organelle isolation resulted in irreversible enzyme inactivation. Inclusion of citrate in the column buffer and gradient medium prevented this loss of activity.

In several respects the castor bean ACL behaves similarly to the well studied enzyme from mammalian sources. Thus the mammalian enzyme is stabilized by adding glycerol and citrate and storing in the frozen state (7), it is strongly inhibited by (-)

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**Table III**  ACL activity in crude extracts from various sources

<table>
<thead>
<tr>
<th>Source</th>
<th>ACL, nmoles/min/seedling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germinating castor bean</td>
<td>195.0</td>
</tr>
<tr>
<td>Ripening castor bean</td>
<td>24.3</td>
</tr>
<tr>
<td>Watermelon</td>
<td>28.0</td>
</tr>
<tr>
<td>Soybean</td>
<td>20.3</td>
</tr>
<tr>
<td>Sweet corn</td>
<td>13.5</td>
</tr>
<tr>
<td>Sunflower</td>
<td>3.8</td>
</tr>
</tbody>
</table>

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**Fig. 4.** Chromatography of a crude extract of germinating castor bean endosperm on agarose (Bio-Gel A-5m). (---): A at 280 nm; (O-O): ATP citrate lyase; (O-O): malate dehydrogenase; (O-O): citrate synthase; (O-O): catalase.

**Fig. 5.** Estimation of the mol wt of ATP citrate lyase from germinating castor bean endosperm by gel filtration on agarose (Bio-Gel A-5m).

**Fig. 6.** Sucrose density gradient separation of organelles from germinating castor bean endosperm. (---): A at 280 nm; (---): sucrose concentration; (O-O): catalase; (A-A): fumarase; (O-O): ATP citrate lyase and (O-O): RuDP carboxylase.
hydroxycitrate and the mol wt of one form of the enzyme (ACL I) is about 400,000 (17). The pH optimum for the castor bean ACL is 7.5, somewhat lower than that from chicken and rat liver (12), but close to the optimum pH 7 for the enzyme from mango fruit (6).

In animal tissues the enzyme is cytosolic (16, 17) and generates acetyl-CoA used by the soluble fatty acid synthetase. In the castor bean, fatty acid synthesis occurs in the plastids (21), as it does in other plant tissues (19). The possibility that part of the ACL activity in the endosperm tissue is cytosolic is not ruled out but the demonstration that the ACL is present in the plastids (Fig. 6) adds considerable weight to the suggestion (6, 8) that in higher plants also the ACL functions to supply the acetyl-CoA precursor for fatty acid synthesis in vivo. There are, however, other possible sources of acetyl-CoA for fatty acid synthesis. As in the ripening castor bean (15) the plastids from the germinating endosperm contain enzymes that could generate pyruvate from sugar phosphates (10) and as shown in Table IV they also contain pyruvate dehydrogenase, although the activity is low compared to that in the mitochondria. The possibility that the plastids can utilize acetate itself as a precursor cannot be ruled out.

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