Identification and Characterization of Mitochondrial Acetyl-Coenzyme A Hydrolase from Pisum sativum L. Seedlings

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ABSTRACT

Mitochondria from Pisum sativum seedlings purified free of peroxisomal and chlorophyll contamination were examined for acetyl-coenzyme A (CoA) hydrolase activity. Acetyl-CoA hydrolase activity was latent when assayed in isotonic media. The majority of the enzyme activity was found in the soluble matrix of the mitochondria. The products, acetate and CoA, were quantified by two independent methods and verified that the observed activity was an acetyl-CoA hydrolase. The pea mitochondrial acetyl-CoA hydrolase showed a $K_m$ for acetyl-CoA of 74 micromolar and a $V_{max}$ of 6.1 nanomoles per minute per milligram protein. CoA was a linear competitive inhibitor of the enzyme with a $K_i$ of 16 micromolar. The sensitivity of the enzyme to changes in mole fraction of acetyl-CoA suggested that the changes in the intramitochondrial acetyl-CoA/CoA ratio may be an effective mechanism of control. The widespread distribution of mitochondrial acetyl-CoA hydrolase activity among different plant species indicated that this may be a general mechanism in plants for synthesizing acetate.

The origin of plastid acetyl-CoA for fatty acid synthesis has been the subject of considerable controversy in the last few years (15). One mechanism proposed for generating plastidial acetyl-CoA, via the acetate activating enzyme acetyl-CoA synthetase, was first identified by Miller and Bonner (18). Acetyl-CoA synthetase has since been partially purified from potato tubers (8) and spinach leaves (28). Acetyl-CoA synthetase is specifically localized in the chloroplast of spinach (11), the exclusive site of fatty acid synthesis (20).

Despite the widespread distribution of acetyl-CoA synthetase among different plant species, acetyl-CoA synthetase as the primary source of plastidial acetyl-CoA is disputed because of the limited information on the origin of its substrate, acetate and the occurrence of chloroplast PDC (3). Although acetate was the preferred exogenous substrate for fatty acid synthesis is isolated spinach chloroplast (23) pyruvate was favored in chloroplasts of Sinapis alba L. (12). Endogenous levels of acetate measured in leaf tissue from several plant species range from 0.07 to 1 mM (11, 14, 26). Yet, a general mechanism(s) for producing acetate has not been adequately defined.

Stumpf and coworkers (13, 19) identified an acetyl-CoA hydrolase activity associated with spinach leaf mitochondria as a source of acetate and proposed a collaboration between mitochondrial acetyl-CoA hydrolase and chloroplastic acetyl-CoA synthetase as a mechanism for generating acetyl-CoA within the chloroplast. The mitochondrial acetyl-CoA would be generated by PDC action. The acetate formed would diffuse out of the mitochondria and reesterify with CoA-SH by chloroplastic acetyl-CoA synthetase. Givan and Hodgson (7) questioned the general presence of this compartmentation scheme in plants when they were unable to detect acetyl-CoA hydrolytic activity in pea mitochondria. The only acetyl-CoA hydrolase they observed was OAA dependent and attributed to citrate synthase. As a result, they argued in favor of an alternative mechanism for generating plastidial acetyl-CoA through the action of chloroplast isozyme of PDC (3).

Several lines of evidence, however, support the presence of an 'acetate pathway' in pea leaves. First, acetate was measured in pea leaf extracts at a concentration comparable to that observed in spinach leaf extracts (26). Second, acetate incorporation studies (24) and, more recently, enzyme localization studies established acetyl-CoA synthetase presence in pea chloroplast that has activity comparable to chloroplast PDC activity (3, 26). These observations suggest that either another source of acetate is present or acetyl-CoA hydrolase is inactivated during isolation or the assay system used by Givan and Hodgson (7) to measure acetyl-CoA hydrolytic activity was inadequate.

Considering these possible alternatives the last explanation seemed reasonable when we examined the assay conditions. Liedvogel and Stumpf (13), using a radioisotope assay, measured an average specific activity of 47 nmol/h/mg protein for spinach mitochondrial acetyl-CoA hydrolase. At these rates and the protein concentration used by Givan and Hodgson (7), an incubation time of greater than 30 min was required to detect acetyl-CoA hydrolytic activity. Givan and Hodgson (7) incubated the mitochondrial extract no longer than 10 min. In light of this observation, we reexamined pea mitochondria for acetyl-CoA hydrolytic activity using the more sensitive radioisotope assay. Herein, we establish that acetyl-CoA hydrolase activity is present in pea mitochondria and has properties similar to that described for spinach acetyl-CoA hydrolase. The pea enzyme can be primarily regulated by changes in the mole fraction of acetyl-CoA/[acytel-CoA]/[CoA-SH] + [acytel-CoA]. Metabolic conditions in the mitochondria favoring acetate formation are described.

1 This research was supported by the Missouri Agricultural Experiment Station and National Science Foundation grant DMB-8506473. This is journal report No. 10,916 from the Missouri Agricultural Experiment Station.
2 Present address: Plant Science Department, University of Arizona, Tucson, AZ 85721.
3 Abbreviations: PDC, pyruvate dehydrogenase complex; DTNB, 5,5'-dithiobis-(2-nitrobenzoate); OAA, oxaloacetate.
MATERIALS AND METHODS

Chemicals and Reagents

The Aminex HPX-87H ion exclusion column was purchased from Bio-Rad. The Microsorb C18 column was from Ranin. Percoll was obtained from Pharmacia, Inc. The acetate determination kit was from Boehringer Mannheim. Acetyl-CoA, CoA, ATP, and NAD⁺ were purchased from P-L Biochemical. [1-¹⁴C]Acetate was obtained from Amersham. [1-¹⁴C]Acetyl-CoA was obtained from Research Products International Corporation. [1,5-¹⁴C]Citrate and [2-¹⁴C]pyruvate were from New England Nuclear. Scintiverse E was purchased from Fisher. All other biochemicals were purchased from Sigma Chemical Company.

Plant Material

Pea seeds (Pisum sativum L. var Little Marvel) were soaked in running tap water overnight and planted in vermiculite-filled flats at a seed density of approximately 100 seeds per flat. The peas were grown in a growth chamber at 18°C and 10 h of illumination at 250 μE m⁻² s⁻¹ until the pea seedlings were 14 d old.

Acetyl-CoA Hydrolase Assay

Acetyl-CoA hydrolase activity was measured by the radioisotope assay described by Liedvogel and Stumpf (13). The reaction mixture in a total volume of 100 μL contained: 50 mM Tricine-KOH (pH 7.5), 0.5 mM MgCl₂, and 200 μM [¹⁴C] acetyl-CoA (4 μCi/μmol). Acetyl-CoA hydrolase activity was alternatively measured by the DTNB assay (13). The assay medium contained in a total volume of 200 μL: 50 mM Tricine (pH 7.5), 0.5 mM acetyl-CoA, and 100 μM DTNB.

Mitochondria Isolation

Mitochondria were isolated from 14 d old pea seedlings using the method of Fang et al. (6). After isolation, the mitochondria were stored at −20°C in RMB buffer until assayed for acetyl-CoA hydrolase activity. The RMB buffer contained 0.3 m mannitol, 20 mM Tes (pH 7.2), 1 mM EDTA, 2 mM MgCl₂, 0.1% (w/v) defatted BSA, and 2 mM DTT.

To enrich for acetyl-CoA hydrolase, the mitochondria were thawed and centrifuged 1 h at 100,000g in a Beckman ultracentrifuge using a TLA 100.2 rotor. The resulting supernatant was dialyzed overnight against 20 mM Tricine (pH 7.5), 0.5 mM MgCl₂, and 20% glycerol. It was this preparation that was used for characterization of acetyl-CoA hydrolase activity.

Mitochondria were isolated from spinach leaf, potato tuber, castor bean endosperm (3 d postgermination), cucumber cotyledons (3 d postgermination), and 10 d old maize and millet seedlings as described by Zeiher (29).

Subcellular Distribution of Acetyl-CoA Hydrolase

Pea leaf homogenates were fractionated by differential centrifugation using the procedure described for pea mitochondrial isolation up to the first discontinuous Percoll gradient step with minor modifications (6). The modifications were both the 1,000g pellet and 18,000g supernatant were retained. The 1,000g pellet was resuspended in 50 mM Tricine (pH 7.8), 1 mM EDTA, 3.5 mM MgCl₂, 13.5 mM 2-mercaptoethanol, and 0.2% (w/v) BSA. Aliquots of the unfractionated homogenate, 1,000g pellet, 18,000g pellet, and 18,000g supernatant were assayed for acetyl-CoA hydrolase and fumarase activity and measured for proteins.

Identification of [¹⁴C]-Reaction Products

The dialyzed 100,000g supernatant from frozen/thawed pea mitochondria was assayed for acetyl-CoA hydrolase activity in a standard reaction mixture as described for the radioisotope assay except the total reaction mixture was increased to 200 μL. The reaction was quenched after a 10 or 30 min incubation with 20 μL of glacial acetic acid to which 220 μL of 0.01 N HCl and 44 μL of activated charcoal were added. The reaction mixture was agitated overnight and then centrifuged at 15,000g to pellet the activated charcoal. An Aminex HPX-87H ion exclusion column attached to a Waters HPLC system was used to separate the [¹⁴C]-reaction products in the clarified supernatant. The mobile phase was 0.018 % H₂SO₄ and 20% acetonitrile pumped at a flow rate of 0.5 mL/min and a pressure of 4100 psi. A 198 μL aliquot of the clarified supernatant was injected onto the column and fractions of approximately 0.21 mL were collected and counted in a Beckman LS 7000 scintillation counter. Standards of 200 μM [¹⁴C]pyruvate (4 μCi/μmol), 200 μM [¹⁴C]citrate (4 μCi/μmol), and 200 μM [¹⁴C]acetate (4 μCi/μmol) in 50 mM Tricine (pH 7.5) and 0.5 mM MgCl₂ were injected separately on to the column after adding 4% (v/v) glacial acetic acid. The elution profiles of the [¹⁴C] standards were compared with the elution profiles of the unknowns for identification of the reaction products. The volatilization of the [¹⁴C] product in the clarified supernatant was measured simultaneously using the volatilization procedure described by Liedvogel and Stumpf (13).

Substrate/Product Stoichiometry of Acetyl-CoA Hydrolase Reaction

The dialyzed 100,000g supernatant of frozen/thawed pea mitochondria was incubated in a 1.0 mL reaction mixture containing a 50 mM KH₂PO₄ (pH 7.5), 0.5 mM MgCl₂, and 200 μM acetyl-CoA. The reaction was quenched with 80 μL of 10% HClO₄ after a 30 min incubation, then neutralized to pH 5.2 by addition of 50 μL of KHCO₃, and centrifuged at 15,000g for 10 min to pellet precipitated proteins.

The acetate concentration of the clarified supernatant was determined using the acetate kit of Boehringer Mannheim. This kit utilized an enzyme-linked assay containing acetyl-CoA synthetase, citrate synthase, and malate dehydrogenase. With this assay the acetate consumed was coupled to the reduction of NAD⁺ measured spectrophotometrically at 340 nm.

The CoA and acetyl-CoA concentration in the clarified supernatant was measured by HPLC (9) using a Microsorb C18 column (250 × 4.6 mm; 5 μm beads). The mobile phase was 220 mM KH₂PO₄ (pH 4.0) and 12% methanol at a flow
rate of 1.0 mL/min and a pressure of 2000 psi. The eluant was monitored at 254 nm and 0.04 mm thymidine was included as an internal standard. The retention times of thymidine, CoA, and acetyl-CoA were 10, 12, and 37 min, respectively. The concentrations of CoA and acetyl-CoA in the reaction mixture were determined by comparing the measured absorbances to absorbances of CoA and acetyl-CoA standards.

Kinetic Analysis

Data generated from product inhibition experiments were fitted to competitive, noncompetitive, and uncompetitive equations using the least squares method and computer program described by Cleland (4). The pattern reported was the pattern which best fit the data to the equation.

Other Analytical Methods

Proteins were determined according to the method of Bradford (2) using BSA as standard. Cyt c oxidase and fumarase activity were measured using the assay described by Tolbert et al. (25) and Zeiher (29), respectively.

RESULTS

Localization of Acetyl-CoA Hydrolase in Pea Mitochondria

The purified mitochondria were essentially free of Chl (0.04% contamination) and peroxisomal (0.2% contamination) contamination and were greater than 95% intact as determined by the latency of Cyt c oxidase (5). The mitochondria exhibited high rates of respiration (154 nmol/min/mg protein) with pyruvate plus sparker malate as substrate and reasonable respiratory control (3.41). Thus, the mitochondria purified in this manner were intact and showed metabolic competence. It was these mitochondria which were used as the starting material for isolation and subsequent characterization of acetyl-CoA hydrolase.

Acetyl-CoA hydrolase activity associated with pea mitochondria was latent. Little acetyl-CoA hydrolase activity was observed in intact mitochondria (Table 1). Breaking the mitochondria by freeze/thawing increased acetyl-CoA hydrolase activity fourfold. Separation of the broken mitochondria into matrix and membrane fractions by ultracentrifugation at 100,000g for 1 h showed that 90% of the total mitochondrial acetyl-CoA hydrolase activity was associated with the matrix fraction.

Subcellular fractionation of a pea leaf cell by differential centrifugation was performed to determine the proportion of acetyl-CoA hydrolase activity in the mitochondria relative to the unfractionated homogenate. Approximately 20% of the total acetyl-CoA hydrolytic activity and 86% of the mitochondrial marker fumarase activity were equally divided between the 1,000g pellet and 18,000g pellet. For each enzyme the total recovery in each of the different fractions accounted for at least 90% of the total activity observed in the unfractionated homogenate.

Acetyl-CoA utilization by the matrix fraction increased with time (Fig. 1A) and protein concentration (Fig. 1B). No acetyl-CoA hydrolytic activity was observed with boiled mitochondrial matrix. Givan and Hodgson (7) reported that the only acetyl-CoA hydrolytic activity they observed in pea leaf mitochondria was OAA dependent and attributed to citrate synthase. To prevent possible interference of citrate synthase and endogenous levels of OAA in the matrix fraction, the

| Table 1. Acetyl-CoA Hydrolase Activity in Different Subfractions of Pea Leaf Mitochondria |
|---------------------------------------------|-------------------|-------------------|-------------------|-------------------|
| Fraction                        | Specific Activity | Total Activity Yield | %                |
|---------------------------------|-------------------|----------------------|-------------------|-------------------|
| Intact mitochondria             | 30.20             |                      |                   |                   |
| Broken mitochondria             | 119.97            | 1323                 | 100               |                   |
| Mitochondria matrix             | 305.60            | 1186                 | 90                |                   |
| Mitochondria membranes          | 15.90             | 107                  | 8                 |                   |
| * 100,000g supernatant.         |                   |                      |                   |                   |
| b 100,000g pellet.              |                   |                      |                   |                   |

Figure 1. A, Time dependence of acetyl-CoA utilization by the 100,000g supernatant before and after dialysis of the 100,000g supernatant against 20 mM Tricine (pH 7.5), 0.5 mM MgCl₂, and 20% glycerol. The amount of protein per reaction was 98 μg protein for the 100,000g supernatant not dialyzed and 102 μg protein for the dialyzed 100,000g supernatant. As a control, acetyl-CoA utilization with boiled 100,000g supernatant was measured after a 60 min incubation. B, Acetyl-CoA utilization by the 100,000g supernatant as a function of protein concentration. The 100,000g supernatant was dialyzed prior to measuring acetate production. The incubation time per reaction was 10 min.
100,000g supernatant was dialyzed overnight against 50 mM Tricine (pH 7.5), 0.5 mM MgCl₂, and 20% glycerol. Dialysis had no effect on acetyl-CoA hydrolytic activity. The acetyl-CoA hydrolytic activity in the dialyzed 100,000g supernatant was stable with no loss of activity observed when the 100,000g supernatant was stored at 4°C for 10 d.

Identification of the Reaction Products of Acetyl-CoA Hydrolase

Acetate produced by acetyl-CoA hydrolase was measured indirectly by measuring the volatilization of the radiolabeled product in the reaction mixture at room temperature. Identification of the volatile product was therefore necessary to establish that the observed activity was indeed acetyl-CoA hydrolase. The radiolabeled reaction products from [¹⁴C]acetate-CoA incubation with mitochondrial matrix fraction were analyzed by HPLC using an Aminex Ion Exclusion HPX-87H column. As presented in Figure 2A, this column well separated [¹⁴C]citrate, [¹⁴C]pyruvate, and [¹⁴C]acetate with retention times of 12, 14, and 18 min, respectively. Injection of the reaction mixture onto the column after removal of [¹⁴C]acetate-CoA with activated charcoal resulted in a single [¹⁴C]labeled peak which had the same retention time (18 min) as the [¹⁴C]acetate standard (Fig. 2B). The amount of radioactivity in the acetate peak was time dependent. Boiling the mitochondrial matrix prior to incubation with [¹⁴C]acetate-CoA prevented accumulation of radiolabeled in the acetate peak. By comparing the amount of radioactivity volatilized (acetate) in the standard assay with that which was measured in the acetate peak, greater than 85% of radioisotope volatilized was estimated to be acetate.

The stoichiometry of substrates and products of the observed acetyl-CoA hydrolase reaction was examined. The disappearance of acetyl-CoA and production of CoA were measured by HPLC using a reverse-phase C₁₈ column and the production of acetyl-CoA was measured with the acetate kit from Boehringer Mannheim. After a 30 min reaction time, 60 ± 3 nmol of acetyl-CoA disappeared approximately equal to the 45 ± 7 nmol of acetate and 50 ± 4 nmol of CoA formed.

Properties of Pea Mitochondrial Acetyl-CoA Hydrolase

The properties of the pea enzyme were examined and compared with properties reported for other acetyl-CoA hydrolases. A summary of this comparison is provided in Table II. Pea mitochondrial acetyl-CoA hydrolase showed a broad pH profile with optimal activity between pH 7.5 and 8.5 similar to acetyl-CoA hydrolase from spinach and rat brain (13, 22).

The pea enzyme was unstable at elevated temperatures similar to spinach (13). Preincubation at temperatures ranging between 0 and 100°C for 5 min prior to assaying for acetyl-CoA hydrolase activity at 30°C resulted in a decline in activity at temperatures greater than 40°C with complete loss of activity above 60°C.

The activity of the pea enzyme exhibited typical Michaelis-Menten kinetics. The $K_m$ for acetyl-CoA estimated from Lineweaver-Burke plots was 74 μM and the $V_{max}$ was 6.1 nmol/min/mg protein. The $K_m$ for acetyl-CoA was similar to that reported for the mitochondrial acetyl-CoA hydrolase from hamster brown adipose tissue (1) but was twofold lower than reported for the spinach mitochondrial acetyl-CoA hydrolase (13) and acetyl-CoA hydrolase from rat brain mitochondria (17).

The pea acetyl-CoA hydrolase showed rather broad substrate specificity for different CoA thioesters when CoA formation was measured by the DTNB assay. At a concentration of 500 μM, propionyl-CoA and butyryl-CoA were hydrolyzed more rapidly than acetyl-CoA (Table III). Activity with palmitoyl-CoA was similar to that with acetyl-CoA. Less activity was observed with oleoyl-CoA, succinyl-CoA, and crotonyl-CoA.

Substrate competition experiments indicated that the nonspecificity of this hydrolytic activity toward CoA thioesters was probably not attributable to the presence of several hydro-
lases each specific for a different CoA-thioester. When the enzyme was incubated with 200 µM [14C]acetetyl-CoA in the presence of an equivalent concentration of different unlabeled CoA-thioesters, propionyl-CoA, and butyryl-CoA inhibited the hydrolysis of [14C]acetetyl-CoA to a greater extent than unlabeled acetyl-CoA (Table III). This is in agreement with the substrate specificity observed when the hydrolysis of a CoA thioester was measured directly with the DTNB assay. Of the unlabeled CoA thioesters tested, palmitoyl-CoA and oleoyl-CoA were the most effective inhibitors inhibiting [14C] acetyl-CoA hydrolysis 94 and 93%, respectively. This was in contrast to the results observed when palmitoyl-CoA and oleoyl-CoA hydrolysis were measured directly (Table III) where the rates observed were either equivalent or less than observed for acetyl-CoA. Palmitoyl-CoA and oleoyl-CoA inhibition thus appears to be nonspecific possibly a detergent effect caused by the long hydrocarbon chain.

Both the spinach and rat brain enzyme were also not specific for acetyl-CoA. The spinach enzyme hydrolyzed acetyl, butyryl, and propionyl-CoA with equal efficiency with less hydrolysis observed with palmitoyl-CoA (13). The rat brain enzyme hydrolyzed acetyl, propionyl, butyryl, acetocacetyl, and succinyl-CoA, and exhibited Kₘ for these respective CoA-thioesters of less than 100 µM. Palmitoyl-CoA was not a substrate for the rat brain enzyme (22).

**Regulatory Properties of Pea Mitochondrial Acetyl-CoA Hydrolase**

CoA-SH is an effective inhibitor of pea mitochondrial acetyl-CoA and 0.1 mM CoA-SH inhibited acetyl-CoA hydrolase 61%. Acetate at a 100-fold higher concentration by contrast had no effect on acetyl-CoA hydrolase activity. Double reciprocal plots of velocity versus acetyl-CoA concentration at different fixed concentrations of CoA showed the inhibition as competitive (Fig. 3). The Kₘ for CoA was 16 µM. A similar CoA inhibition was observed for the spinach enzyme (13). However, Liedvogel and Stumpf (13) reported a noncompetitive inhibition by CoA-SH with acetyl-CoA as the variable substrate. The differences in the inhibition patterns shown here and observed for the spinach enzyme are probably a reflection of the relative purity of the two enzymes.

### Table II. Comparison of the Properties of Mitochondrial Acetyl-CoA Hydrolases from Different Organisms

<table>
<thead>
<tr>
<th>Property</th>
<th>Source of Mitochondrial Acetyl-CoA Hydrolase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pea leaf</td>
</tr>
<tr>
<td>Location</td>
<td>Matrix</td>
</tr>
<tr>
<td>pH optimum</td>
<td>7.5–8.5</td>
</tr>
<tr>
<td>Temperature instability</td>
<td>&gt;40°C</td>
</tr>
<tr>
<td>Kₘ (Acetyl-CoA, µM)</td>
<td>74</td>
</tr>
<tr>
<td>Vₘₐₓ (nmol/min mg protein)</td>
<td>6.1</td>
</tr>
<tr>
<td>Substrate specificity</td>
<td>C₂ = C₄ &gt; C₂ = C₁₈</td>
</tr>
</tbody>
</table>


### Table III. Substrate Specificity of Pea Leaf Mitochondrial Acetyl-CoA Hydrolase

<table>
<thead>
<tr>
<th>Thioester</th>
<th>Specific Activity*</th>
<th>Rate Relativeb to Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol CoA formed/ min/mg protein</td>
<td>(1.0)</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>9.60 ± 1.08</td>
<td>0.59</td>
</tr>
<tr>
<td>Propionyl-CoA</td>
<td>57.91 ± 4.62</td>
<td>0.35</td>
</tr>
<tr>
<td>Butyryl-CoA</td>
<td>56.03 ± 4.69</td>
<td>0.33</td>
</tr>
<tr>
<td>Palmitoyl-CoA</td>
<td>10.29 ± 0.70</td>
<td>0.06</td>
</tr>
<tr>
<td>Oleoyl-CoA</td>
<td>6.58 ± 0.93</td>
<td>0.07</td>
</tr>
<tr>
<td>Succinyl-CoA</td>
<td>3.89 ± 0.39</td>
<td>0.51</td>
</tr>
<tr>
<td>Crotonyl-CoA</td>
<td>6.94 ± 0.29</td>
<td>0.62</td>
</tr>
</tbody>
</table>

* Acetyl-CoA hydrolase was assayed by the DTNB assay described in "Materials and Methods." The respective thioesters were substituted for acetyl-CoA at a final concentration of 0.5 mM in a standard reaction mixture. ° Unlabeled CoA thioesters were added to a standard assay of [14C]acetetyl-CoA hydrolisis at a final concentration of 0.2 mM.

The influence of the mole fraction (i.e. [acetetyl-CoA]/CoA-SH) + [acetetyl-CoA]) of acetyl-CoA to the total CoA pool on acetyl-CoA hydrolase was examined (Fig. 4). When acetyl-CoA hydrolitic activity was measured at different mole fractions of acetyl-CoA keeping the total CoA pool constant at 250 µM, a concentration comparable to that observed in isolated pea mitochondria (RJA Budde, TK Fang, DD Randall, unpublished data), a curvilinear response was observed as the mole fraction approached unity. Acetyl-CoA hydrolase activity was inhibited 50% when approximately 25% of the total CoA was in the acetylated form.

### Distribution of Acetyl-CoA Hydrolase Among Different Plant Species and Different Plant Tissues

Mitochondria were isolated from leaf tissue of several plant species and examined for acetyl-CoA hydrolytic activity. As presented in Table IV, acetyl-CoA hydrolase activity was observed in leaf mitochondria of all plant species examined at rates comparable or greater than rates observed for the spinach enzyme. The presence of acetyl-CoA hydrolase was not specific to leaf tissue since activity was also observed in mitochondria isolated from potato tubers, endosperm of ger-
mitochondria, and cotyledons of germinating cucumber seeds.

DISCUSSION

This report clearly demonstrates the presence of an acyl-CoA hydrolytic activity in pea mitochondrial matrix. Although this enzyme possesses a broad substrate specificity (see Table III), we have designated the activity as an acetyl-CoA hydrolase based on the conclusion that acetyl-CoA is the primary physiological substrate available to the enzyme. To our knowledge formation of middle-chain and long chain thioesters by acyl-CoA synthetase(s) has not been demonstrated in plant mitochondria and propionyl-CoA and butyryl-CoA have not been found to be common mitochondrial metabolites. Palmitoyl carnitine acyltransferase as a source of palmitoyl-CoA was recently identified in etiolated pea cotyledon mitochondria (27). Wood et al. (27) provided evidence that the palmitoyl-CoA formed is oxidized to acetyl-CoA by \( \beta \)-oxidation. Acetyl-CoA, on the other hand is readily formed by mitochondrial PDC and is presumably the main short chain CoA thioester in mitochondria. In agreement with Liedvogel and Stumpf's conclusion (13), the primary physiological role of this enzyme will therefore be defined by the matrix location of the enzyme. Designating the activity characterized in this report as an acetyl-CoA hydrolase is in agreement with previous literature reports (1, 13, 17).

Measurement of acetate formation rather than CoA formation and utilization of dialyzed mitochondrial extracts as a source of the enzyme eliminated any possibility that the hydrolytic activity observed was due to the presence of citrate synthase and endogenous levels of OAA. The authenticity of the acetyl-CoA hydrolase reaction was confirmed when the reaction products acetate and CoA were measured and shown to be produced in the predicted 1:1 stoichiometry of an acetyl-CoA hydrolase reaction. These results together with the similarities in properties of the pea enzyme to other acetyl-CoA hydrolases strongly supports the pea mitochondrial enzyme as a true acetyl-CoA hydrolase.

The maximal rates observed for the pea mitochondrial enzyme were 6.1 to 9.1 nmol/min/mg protein which are comparable to the activity observed for acetyl-CoA synthetase in pea chloroplasts (26). As incorporation of labeled acetate into lipids by isolated pea chloroplast is about one-fifth of the maximal rates of acetyl-CoA synthetase, the observed acetyl-CoA hydrolase activity would be sufficient to provide the necessary acetate for acetyl-CoA synthetase and fatty acid synthesis. Moreover, Budde et al. (unpublished data) measured acetyl-CoA levels in active respiring mitochondria which ranged between 150 and 300 \( \mu \)M. As these values are similar to the \( K_m \) measured for acetyl-CoA hydrolase (74–150 \( \mu \)M; Table III; [9]) and well above the \( K_m \) for citrate synthase (11–15 \( \mu \)M; [7]) suggests that there would be sufficient substrate for acetyl-CoA hydrolase to provide acetate for fatty acid synthesis. These results are consistent with the compartmentation scheme formulated by Stumpf and coworkers (13, 19) in spinach leaves and extends this observation to pea leaf tissue.

Interestingly, the majority of acetyl-CoA hydrolytic activity could not be accounted for by the mitochondrial marker fumarase. This suggests that there may be another possible source of acetate within the plant cell. Evidence for a non-mitochondrial acetyl-CoA hydrolytic activity will be the subject of a separate report.

The failure of Givan and Hodgson (7) to detect acetyl-CoA hydrolase in pea mitochondria may be the result of several contributing factors. First, the acetyl-CoA concentration (56 \( \mu \)M) in their reaction mixture was less than the \( K_m \) for acetyl-CoA measured for pea (\( K_m = 74 \mu \)M; Table III) and spinach (\( K_m = 150 \mu \)M; [13]) mitochondrial acetyl-CoA hydrolase. Second, the protein concentration of their reaction mixture was 26-fold less than we used and 37-fold less than Liedvogel and Stumpf (13) used for the DTNB assay. These two factors along with the short incubation times used by Givan and Hodgson (7) would prevent (in the absence of OAA) enough accumulation of CoA-SH to be detected by the DTNB assay. As a result, the low acetyl-CoA and protein concentrations in their reaction mixture favored the more active citrate synthase (7).

The effect of changing the mole fraction of acetyl-CoA on acetyl-CoA hydrolase activity in the 100.000g supernatant. The total CoA pool was 250 \( \mu \)M. Each point represents mean of duplicate samples. The amount of protein per reaction was 48 \( \mu \)g.
The pea mitochondrial acetyl-CoA hydrolase is quite sensitive to product inhibition by CoA-SH. This is predicted by the significantly lower \( K_m \) for CoA (16 \( \mu \)M) compared to the \( K_m \) for acetyl-CoA (\( K_m = 74 \mu \)M). Control of the enzyme by changes in the CoA-SH/acetyl-CoA ratio is thus suggested. A similar control mechanism has been described for acetyl-CoA hydrolase from mitochondria of hamster brown adipose tissue (1).

Factors affecting the intramitochondrial mole fraction of acetyl-CoA and hence acetate formation include acetyl-CoA synthase and the utilization of acetyl-CoA by the citric acid cycle. Plant mitochondrial acetyl-CoA is primarily formed by the oxidative decarboxylation of pyruvate by PDC. PDC is highly regulated by reversible phosphorylation and product inhibition (21). Under metabolic conditions where PDC is in the active state (dephosphorylated), PDC and hence acetyl-CoA formation will be controlled by the concentration of its products NADH and acetyl-CoA (21).

The demands of the citric acid cycle on the mitochondrial acetyl-CoA pool will limit the acetyl-CoA available for hydrolysis. Citrate synthetase's higher affinity for acetyl-CoA \( [K_m \text{ (acetyl-CoA)} = 11-15 \mu \text{M}; \text{ref. 7] compared to acetyl-CoA hydrolase} \ [K_m \text{ (acetyl-CoA)} = 74-150 \mu \text{M}; \text{Table III and ref. 13}] \) suggests preferential channeling of acetyl-CoA into the citric acid cycle. Murphy and Stumpf (19) showed greater incorporation of \( [1^{14} \text{C}] \text{acetyl-CoA} \) into citrate (84%) compared to acetate (15%) in spinach mitochondrial extracts. As a result, the contribution of the mitochondrial acetyl-CoA hydrolase to the cellular acetate pool is expected to be small except under conditions when the ratio of PDC to citrate synthase activity is greater than one.

The flux through citrate synthase will be primarily controlled by the levels of OAA since the OAA concentration in plant mitochondria (<1 \( \mu \)M; [5]) is less than the \( K_m \) value for OAA measured for citrate synthase (1-5 \( \mu \)M; [7]). The intramitochondrial OAA concentration is largely determined by the malate concentration and the NADH/NAD\(^+\) ratio because of the unfavorable equilibrium of the malate dehydrogenase reaction (5). Budde et al. (unpublished data) showed with pea mitochondria oxidizing pyruvate plus malate as substrate that an increase in malate concentration from 0.1 to 0.5 mM decreased the intramitochondrial mole fraction of acetyl-CoA from 0.85 to 0.62. A similar decrease in mole fraction of acetyl-CoA inhibited acetyl-CoA hydrolase 46%. Consequently, conditions of limited OAA and ample pyruvate would favor acetyl-CoA accumulation and hence acetate formation. Similar metabolic conditions promote ketogenesis in rat liver mitochondria (16). Formation of acetate in plants may be analogous to ketogenesis in animals as a mechanism for redistributing carbon to other cellular compartment(s) under conditions of limited utilization of acetyl-CoA by the citric acid cycle. Such a role for acetyl-CoA hydrolase has been proposed for acetyl-CoA hydrolase in rat heart mitochondria where ketogenesis is low (10).

Acetyl-CoA synthetase is found in all plants examined to date in both photosynthetic and nonphotosynthetic tissue (8, 18, 26, 28). Acetyl-CoA hydrolase also appears to have a similar widespread distribution. We found acetyl-CoA hydrolytic activity in mitochondria isolated from several plant species and plant tissues with rates similar or greater than that reported for the spinach enzyme (13). The ubiquitous presence of both acetyl-CoA hydrolase and acetyl-CoA synthetase thus suggests that the coupling of these two enzymes may be a common 'pathway' in plants from acetate producing sites to acetate utilizing sites.

In summary, we have isolated an acetyl-CoA hydrolase in the matrix fraction of pea mitochondria. The activity observed was verified as an acetyl-CoA hydrolase by quantifying its products and by comparison of its properties to other described acetyl-CoA hydrolases. The identification of acetyl-CoA hydrolase in leaf tissue of plant species in addition to spinach suggests that this is a common mechanism plants evolved for producing acetate for acetyl-CoA synthetase and fatty acid synthesis.

ACKNOWLEDGMENTS

The authors thank Dr. Curt Givan for his advice and discussions of the results, and Nancy R. David for assistance with organelle isolation.

LITERATURE CITED


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<tr>
<th>Table IV. Acetyl-CoA Hydrolase Activity in Mitochondria Isolated from Different Plant Species</th>
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<td>Plant Species</td>
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<td>Castor Bean (germinating)</td>
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<td>Cucumber (germinating)</td>
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