ROLE OF THE MITOCHONDRIA IN THE CONVERSION OF 1-AMINOCYCLOPROPANE 1-CARBOXYLIC ACID TO ETHYLENE IN PLANT TISSUES

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Intact plant mitochondria, isolated from climacteric (Lycopersicon esculentum, Mill., tomato) or non-climacteric (Solanum tuberosum, L., potato) tissues, and purified on Percoll density gradients, were unable to convert 1-aminocyclopropane 1-carboxylic acid (ACC) to ethylene. Energization or sonication did not enhance ethylene production. For both tissues, the low activity of ACC conversion found in crude mitochondrial fractions from both tissues was increased by sonication. After mitochondrial purification, this activity was located on top of the gradient together with the microsomal membrane fraction containing a high lipoxygenase activity. Addition of exogenous lipoxygenase and linoleic acid to isolated tomato or potato mitochondria greatly enhanced ACC conversion (to approx. 300 pmol h⁻¹ mg⁻¹ protein). Direct measurements of ACC uptake by mitochondria indicated that ACC uptake is not dependent on energization.

Key words: mitochondria; ethylene biosynthesis; lipoxygenase

Introduction

Previous workers have reported contradictory results from studies examining the possible involvement of mitochondria in the biosynthesis of ethylene [1,2]. Recently, Winkler and Apelbaum [3-5], using ACC, the immediate precursor for biosynthetic ethylene formation [6,7] showed ACC-dependent ethylene production by mitochondria isolated from etiolated pea seedlings. These authors also suggested, based on sonication experiments, that mitochondrial ACC uptake was the step limiting ethylene production.

It has recently been shown, however, that ACC is converted to ethylene in extracts containing lipoxygenase and lipid [8]. Because lipoxygenase is a non-mitochondrial enzyme [9,10] and largely soluble, it seems possible that ethylene production by impure mitochondria may be associated with lipid oxidation.

The present study was undertaken to elucidate the role of mitochondria in ACC-dependent ethylene formation. Mitochondria were isolated from two different tissues (climacteric and non-climacteric), and the activity of ethylene production was measured in crude and purified mitochondrial fractions. The effects of modifying mitochondrial structure by sonication or the addition of lipoxygenase and free fatty acids (linoleic acid) were also investigated. Direct measurements of ACC uptake were carried out to determine if this precursor was actively accumulated by mitochondria.

Materials and methods

Chemicals. Lipoxygenase purified from Soybean (Fraction V), linoleic acid (grade III) and ACC were purchased from Sigma. [¹⁴C]-
ACC was obtained from the Centre d'Etudes Atomiques (France).

**Preparation of mitochondria and microsomes.** Mitochondria were prepared from either potato tubers (S. tuberosum, L.) or tomato fruits (L. esculentum, Mill.). Potato mitochondria were isolated as described previously [11]. Tomato mitochondria were isolated from fruits at a stage close to the climacteric ('breaker') stage using the procedure described by Moreau and Romani [12]. Crude mitochondrial fractions obtained by differential centrifugation were purified on self-generating Percoll gradients using 25% (v/v) Percoll (Pharmacia) in the starting solution containing 0.25 M sucrose and 50 mM Tris--HCl (pH 7.3). After centrifugation at 27 000 × g for 35 min, three fractions were collected. The light fraction (ρ = 1.05) contained microsomal membranes, the heavier fraction (ρ = 1.07) corresponded to purified mitochondria and the pellet (ρ > 1.10) consisted of peroxisomes [10]. A proper microsomal fraction was obtained by centrifugation at 100 000 × g for 30 min (Spinco, rotor 60) of the supernatant fraction obtained from the crude mitochondrial pellet. All of these fractions were resuspended in the assay medium used to determine the ACC conversion.

**Measurement of ACC conversion.** The standard assay medium contained 0.3 M mannitol, 0.1 mM MnCl₂, 10 mM ACC and 10 mM morpholinoethane sulfonic acid (MOPS). The reaction was carried out at either pH 7.0 or 8.0. There were approx. 2–5 mg of mitochondrial protein per ml of the incubation medium. The reaction mixture was placed in a 25-ml glass tube, and the reaction was started by adding ACC. Each test tube was closed with a rubber septum and incubated in a shaking bath at 25 or 28°C for 2 h. Ethylene was measured in 1.0-ml samples of the air in the test tubes using an Intersmat gas chromatograph equipped with a flame ionization detector and a 90-cm column of 100 mesh Al₂O₃ held at 70°C.

**Measurement of lipoxygenase activity.** Determination of lipoxygenase activity of fractions from Percoll gradients was measured according to Rustin et al. [13].

**Oxygen uptake.** Integrity of mitochondrial fractions was evaluated by measuring their oxygen uptake at 25°C with a Clark-type oxygen electrode [12]. For these measurements, 10 mM inorganic phosphate (final concentration) and 1 mg ml⁻¹ bovine serum albumin were added to the standard medium.

**Determination of ACC uptake.** Uptake of ACC by mitochondria was assayed by measuring the incorporation of [2,3-¹⁴C]ACC, in 1 ml of standard medium supplemented with 10 mM ACC (final concentration) and 3 μCi [¹⁴C]ACC. Mitochondria were filtered on Millipore membranes (0.45 μm) then rapidly washed three times with 2 ml of standard medium before determination of their radioactivity in a scintillation counter.

**Protein determination.** Protein content of membrane fractions was determined by mineralization and Nesslerization.

**Results**

**ACC conversion in mitochondrial fractions.** Mitochondria were isolated from tissues exhibiting considerable differences in their ability to convert ACC to ethylene. The ACC conversion activity was approx. 850 pmol h⁻¹ mg⁻¹ fresh wt. in breaker tomato tissues and only 30 pmol h⁻¹ mg⁻¹ fresh wt. in potato tissues.

The capacity of crude and Percoll-purified mitochondria isolated from tomato fruit and potato tubers to convert ACC to ethylene was measured at 25°C and at pH 7.0. As shown in Table I, a low conversion activity was found in crude mitochondrial fractions from both tissues. This activity was higher in tomato mitochondria than in potato mitochondria whose conversion activity was less than 10 pmol h⁻¹ (mg protein)⁻¹. Conversion activity was not detectable in purified mitochondrial fractions. Similar results were obtained when the assays were repeated at 28°C or at pH 8.0.

When purified mitochondria were incubated in air in the presence of 10 mM succinate and
10 mM ACC for 2 h at 28°C, no significant ACC conversion occurred, even in the presence of nigericin (0.1 μg/mg protein).

Since mitochondria were incubated with ACC for 2 h at 25°C, their oxygen uptake under similar conditions was measured at intervals in order to verify the physiological integrity of mitochondrial preparations during the assays. In a typical experiment with potato mitochondria, the rate of phosphorylating oxidation with succinate as substrate declined gradually from 180 to 140 nmol O₂ min⁻¹ (mg protein)⁻¹. The rate of non-phosphorylating oxidation increased from 45 to 55 nmol O₂ min⁻¹ (mg protein)⁻¹ and the ADP/O ratio fell by approx. 25%. Similar results were obtained with tomato mitochondria whose respiratory control fell from 2.3 to 1.6 during the 2-h experimental period.

Six successive sonication treatments, each for 15 s at 0°C, roughly doubled the bioconversion activity (Table I). However, a similar exposure to sonication did not generate ACC conversion activity in purified mitochondria.

**ACC conversion and lipoxygenase activity in various membrane fractions**

When separation of components of the crude mitochondria were assayed, some conversion of ACC was found in the light fraction, but there was no conversion either in the purified mitochondrial fraction or in the pellet (Table II). Table II also shows that there was a high lipoxygenase activity in the light fraction, whereas there was no detectable activity either in the purified mitochondria or in the pellet.

In contrast, there was no detectable conversion of ACC by the proper microsomal fraction sedimented from the mitochondrial supernatant fluid by high speed centrifugation (data not shown).

**Table I. Effect of sonication on the conversion of ACC to ethylene in crude and purified mitochondria isolated from tomato fruit and potato tuber.**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Ethylene formation (pmol h⁻¹ (mg protein)⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude mitochondria</td>
</tr>
<tr>
<td></td>
<td>Intact</td>
</tr>
<tr>
<td>Tomato</td>
<td>58</td>
</tr>
<tr>
<td>Potato</td>
<td>8</td>
</tr>
</tbody>
</table>
| *ND, not detectable.*

**Table II. Distribution of ACC conversion and lipoxygenase activities in the various membrane fractions separated during purification of potato mitochondria on Percoll gradients.**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>ACC conversion activity (pmol h⁻¹ (mg protein)⁻¹)</th>
<th>Lipoxygenase activity (nmol min⁻¹ (mg protein)⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light fraction</td>
<td>36</td>
<td>2.6</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Pellet</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
| *ND, not detectable.*

**Dependence of ethylene formation on the presence of exogenous lipoxygenase activity**

The ACC-dependent ethylene production activity in either tomato or potato mitochondria incubated for 2 h at 25°C was strongly stimulated by the addition of exogenous lipoxygenase (Table III). The stimulation was greater in potato mitochondria than in tomato mitochondria. In both cases, addition of 5 mM linoleic acid to mitochondria incubated in the presence of lipoxygenase greatly enhanced the conversion of ACC (to approx. 300 pmol h⁻¹ (mg protein)⁻¹). Under the same conditions, but in the absence of a mitochondrial fraction, ethylene production was approx. 3000 pmol h⁻¹ (mg protein)⁻¹ from the lipoxygenase and linoleic acid alone (Table III).

**Uptake of ACC by purified mitochondria**

Purified mitochondria incubated in the presence of labeled [¹⁴C]ACC incorporated 0.5–1.0 mmol ACC (mg protein)⁻¹ (Table IV). Incorporation was complete within 2 min of incubation and remained unchanged for at
Table III. Dependence of ethylene formation on the presence of exogenous lipoxygenase in crude mitochondrial preparations.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Medium</th>
<th>Ethylene formation (pmol h(^{-1}) (mg protein(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ Tomato mitochondria</td>
<td>Standard medium</td>
<td>72</td>
</tr>
<tr>
<td>+ Lipoxygenase (200 units/ml)</td>
<td></td>
<td>90</td>
</tr>
<tr>
<td>+ Lipoxygenase (200 units/ml) + linoleic acid (5 mM) (a)</td>
<td></td>
<td>270</td>
</tr>
<tr>
<td>+ Potato mitochondria</td>
<td>Standard medium</td>
<td>10</td>
</tr>
<tr>
<td>+ Lipoxygenase (200 units/ml)</td>
<td></td>
<td>160</td>
</tr>
<tr>
<td>+ Lipoxygenase (200 units/ml) + linoleic acid (5 mM) (a)</td>
<td></td>
<td>320</td>
</tr>
<tr>
<td>Control (no mitochondria)</td>
<td>Standard medium</td>
<td>3000</td>
</tr>
<tr>
<td>+ Lipoxygenase (200 units/ml) + linoleic acid (5 mM) (a)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(a\)Final concentration.

Least 45 min. The presence of 10 mM succinate did not significantly change the incorporation of ACC.

Discussion

Although the pathway by which methionine is converted to ACC, the immediate precursor to ethylene in plant tissues, has now been well characterized [7], the final step, the bio-oxidation of ACC to ethylene, is still poorly understood. Many plant tissues, particularly those producing significant quantities of endogenous ethylene, rapidly convert applied ACC to ethylene [14]. This conversion has a low \(K_m\) (66 \(\mu\)M) [15] and although it is thought to involve a free-radical attack [16], is highly stereospecific [17].

Attempts to isolate the ACC bioconversion activity from tissues have, so far, been largely unsuccessful. Disruption of the tissue, even in the most gentle manner, almost completely destroys the ethylene-forming enzyme (EFE) activity. Those cell-free preparations that do have the ability to convert ACC to ethylene [18–20] do not have the low \(K_m\), the high activity, or the stereospecificity found in the intact tissue [7]. An EFE activity with characteristics of the activity in vivo has recently been reported in intact vacuoles [21]. The rapid loss of this activity accompanying gentle lysis of the vacuoles is evidence that activity of the EFE relies intimately on structural integrity of cellular membranes.

In several respects the ACC conversion activity demonstrated by Winkler and Apel-

Table IV. Uptake of ACC by purified potato mitochondria after incubation for 45 min at 25°C.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Medium</th>
<th>ACC uptake (nmol (mg protein(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato mitochondria</td>
<td>− succinate</td>
<td>0.99</td>
</tr>
<tr>
<td>+ succinate (10 mM)</td>
<td></td>
<td>0.43</td>
</tr>
<tr>
<td>Potato mitochondria</td>
<td>− succinate</td>
<td>0.55</td>
</tr>
<tr>
<td>+ succinate (10 mM)</td>
<td></td>
<td>0.59</td>
</tr>
</tbody>
</table>
baum [3–5] in mitochondria extracted from pea seedlings resembles the non-specific low activity cell-free preparations reported by other workers [18–20]. Its $K_m$ is relatively high, and the $V_{\text{max}}$ low [22]. The data reported here suggest that the activity is probably an artifact rather than the true EFE occurring in vivo. Conversion rates in crude mitochondrial preparations were very low, whether they were from climacteric tomatoes producing considerable amounts of ethylene, or from potatoes producing very little. When the mitochondria were purified, conversion was no longer detectable unless the contaminating microsomal fraction was present. The demonstration that lipoxygenase activity of fractions from Percoll gradients parallels their ACC conversion activity suggests that the contaminating EFE activity may involve the presence of this enzyme.

ACC is readily oxidized to ethylene by a free-radical generating system [8]. We interpret the EFE-like activity reported by Winkler and Apelbaum [3–5] in mitochondria prepared by differential centrifugation to be the result of peroxidation of membrane lipid in the preparations, possibly in association with contaminating lipoxygenase activity which is known to be very high in pea seedlings [23]. The enhancement in activity by sonication is probably the result of increased rates of peroxidation of the sonicated organelles rather than exposure of specific inner membrane EFE activity.

Acknowledgments

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References