# Ethylene formation from 1-aminocyclopropane-1-carboxylic acid in plant mitochondria

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Mitochondria isolated from apical parts of etiolated pea seedlings convert ACC to ethylene. This conversion is heat-sensitive and largely enhanced when the intact mitochondria are sonicated or when the ionophore nigericin is added to the reaction medium. When the sonicated submitochondiral particles are spun down, almost all of the activity is recovered in the pellet. The results suggest that the ethylene-formation activity is associated with the inner mitochondrial membrane and that transport of ACC into the mitochondrial membrane is a rate limiting step for the reaction. The mechanism of ACC transport across the mitochondrial membrane is discussed.

*Ethylene* *1-Aminocyclopropane-1-carboxylic acid* *Mitochondria* *Submitochondrial particles* *Nigericin*

## 1. INTRODUCTION

The possibility that the mitochondria are involved in the biosynthesis of the plant hormone, ethylene, received considerable attention in the past [1–6]. However, the initial reports on ethylene production catalyzed by the isolated mitochondrial fraction were contradicted in [4–6]. In previous studies methionine, as well as other precursors for ethylene biosynthesis, were used to investigate the ethylene formation capacity of the isolated mitochondria. 1-Aminocyclopropane-1-carboxylic acid (ACC) is an immediate precursor for ethylene synthesis [7] and is formed in the cytoplasm [8]. Thus, isolation of mitochondria from the cytoplasmic components could strip it of the necessary precursors for ethylene formation. This might be part of the reason for the previously low ethylene formation activity attributed to plant mitochondria. Furthermore, previous experiments suggeste that ACC uptake in apple slices is limited [9] and permeability of the mitochondrial membrane to ACC could therefore also be rate-limiting for ethylene formation.

We here show that high activities of ACC-dependent ethylene formation can be demonstrated with a mitochondrial preparation isolated from an ethylene-forming tissue. This activity is associated with the inner mitochondrial membrane. It is also shown that under conditions where easier access of ACC to the inner membrane is allowed, high activities of ethylene formation from ACC can be obtained.

## 2. MATERIALS AND METHODS

Apical parts from 7-day-old etiolated pea seedlings (*Pisum sativum*, var. Kelvedon Wonder) were excised and the mitochondrial fraction was isolated as in [10] with the following modifications: the HEPES buffer was adjusted to pH 7.9, 0.4 M sucrose was used instead of 0.5 M mannitol and the final mitochondrial suspension was washed twice before use. Oxygen uptake by these mitochondrial preparations was measured in the presence of 10 mM succinate as described in [10]

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with a YSI model 53 oxygen electrode, and respiratory control values obtained were 2.0–3.0.

A mitochondrial suspension containing 5–10 mg protein/ml was sonicated for 2 min with a Branson sonifier at maximum output at 0°C. It was then spun down at 10000 × *g* for 10 min at 4°C and the supernatant used as the fraction which contains the submitochondrial particles.

Microsomes were prepared from the homogenized apical parts of 7-day-old pea seedlings which were filtered through cheesecloth and spun down as in [11]. The homogenization medium used for washing and final suspension of the microsomes was the same as that used for the preparation of mitochondria.

The standard assay medium for ethylene included 100 mM Na–HEPES buffer at pH 8.0, 0.1 mM $MnCl_2$, 10 mM ACC and 25 $\mu$l mitochondrial suspension (5–10 mg protein/ml) in a final volume of 1.0 ml. The reaction mixture was placed in a 15-ml glass test tube placed in a shaking bath at 28°C. Each test tube was then closed with a rubber septum and incubated for two periods of 20 min. The reaction was started by the addition of ACC and was found to be linear for at least 1 h. Ethylene was measured in 1.0 ml air samples withdrawn from the gaseous phase of the test tubes. It was then analyzed in a Tracor 560 gas chromatograph equipped with a flame ionization detector and a 90 cm column of 80–100 mesh $Al_2O_3$, and operated at 68°C.

Table 1

Activity of ACC-dependent ethylene formation in different fractions of homogenized apices from etiolated peas

| Fraction | Vol. (ml) | [Protein] (mg/ml) | Units of ethylene formation (nmol/h) |
|---|---|---|---|
| Pellet I | 2.50 | 9.0 | 195 |
| Sonicated pellet I | | | 41 |
| Supernatant I | 92.00 | 11.0 | 1325 |
| Pellet II | 4.66 | 12.5 | 789 |
| Sonicated pellet II | | | 1583 |
| Supernatant II | 88.00 | 6.3 | 646 |
| Pellet III | 2.50 | 12.8 | 578 |
| Sonicated pellet III | | | 556 |
| Supernatant III | 84.00 | 5.5 | n.d. |

Apical sections of etiolated pea shoots (30 g) were homogenized with pestle and mortar in 45.0 ml medium as in section 2. The filtered homogenate was subjected to differential centrifugation sequentially: at 2000 × *g* for 10 min (pellet I, supernatant I), at 10000 × *g* for 10 min (pellet II, supernatant II), and at 130000 × *g* for 1 h, as described in section 2 for microsomal preparation (pellet III, supernatant III). The pellets were suspended in the homogenization medium to a final concentration of 11.0 mg protein/ml, and half of the suspension was exposed to 2 min sonication. The assay of ethylene formation was carried out as described in section 2; n.d., not detectable

## 3. RESULTS AND DISCUSSION

### 3.1. *Ethylene production in different fractions of the homogenized tissue*

Several fractions were obtained by differential centrifugation of the homogenized tissue from etiolated pea seedlings, and their capacity to convert ACC to ethylene was measured. The results are described in table 1. Low activities were found in the first pellet which contains cell wall and cell debris and was spun down at 2000 × *g* for 10 min. Much higer activity was recorded in the supernatant. About 60% of this activity was found in the pellet obtained by centrifugation at 10000 × *g* for 10 min, which contained the mitochondrial fraction. About 40% of this activity was found in the pellet obtained by centrifugation at 130000 × *g* for 60 min, which contained microsomes but it also contained broken mitochondrial vesicles [12]. The ethylene formation activity was found to be ACC-dependent and heat-sensitive (table 2).

### 3.2. *ACC conversion to ethylene by submitochondrial particles*

When the different fractions were exposed to sonication for 2 min, no change in activity was noticed in either the cell debris or the microsomal fraction, but the activity in the mitochondrial fraction was more than doubled. During sonication of the mitochondria the mitochondrial cristae break off in such a manner that the resulting submitochondrial particles are inside out [13,14]. Membrane-bound species which face the matrix in the intact mitochondria, face the medium in submito-

chondrial particles. The increase in the ethylene-producing capacity of the mitochondrial fraction could be attributed either to the release of a soluble ethylene-forming system from the inner mitochondrial lumen or to the exposure of an ethylene-producing system located on the inner mitochondrial membrane. In order to distinguish between these two possibiliteis, the submitochondrial particles were spun down at 100 000 × *g* for 90 min, washed and centrifuged again. Ethylene formation from ACC was then measured both in the submitochondrial pellet and in the supernatant. As can be seen in table 2, the heat-sensitive activity can be fully recovered in the pellet with essentially no activity in the first supernatant.

These results allowed us to conclude that a heat-sensitive ACC-dependent ethylene formation activity is associated with the inner side of the mitochondrial membrane.

### 3.3. *The effect of nigericin on ACC-dependent ethylene formation of mitochondria and submitochondrial particles*

Since ethylene formation activity was shown to be associated with the inner mitochondrial membrane, the lower activities of intact mitochondria could be attributed to the relatively low permeability of the membrane to the ACC. The mode of ACC uptake across biological membranes is not known. Since ACC is a neutral amino acid, a mechanism of transport for ACC across the mitochondrial membrane akin to the one described for neutral amino acids in other systems [15], is considered. The possible involvement of ionic cosubstrates or counter ions in the transport mechanism was tested by studying the influence of ionophores on ACC conversion to ethylene. Nigericin facilitates $H^+ \rightleftarrows K^+$ exchange in the presence of $K^+$. It is known to collapse the proton concentration gradient, having no effect (at low concentrations) on the transmembrane potential gradient [16]. When nigericin is added to the reaction medium containing intact mitochondria, KCl and ACC, an 8–10-fold increase in the rate of ACC conversion to ethylene is obtained. With submitochondrial particles, however, a <2-fold enhancement is observed (fig.1). Similar results were obtained with the ionophore gramicidine (not shown).

The model illustrated in fig.2 describes the working hypothesis for the mechanism of ACC transport into the intact mitochondria. ACC uptake is coupled to proton influx (fig.2A). The flow of positive charges is neutralized by a counterflow of an ionic cosubstrate; i.e., $K^+$. In this model either the influx of $H^+$ or the efflux of the counter ion could be rate-limiting in the ACC uptake process. Hence, these ion movements might be rate-limiting for ACC conversion to ethylene since this reaction takes place at the inner side of the mitochondrial membrane. When nigericin is added (fig.1,2B), both $H^+$ influx and $K^+$ efflux are facilitated. As a result, the concomitant uptake of ACC into the inner mitochondrial lumen is faster and higher rates of ethylene formation are observed. Submitochondrial particles exhibit a relatively low enhancement by nigericin owing to the fact that the inner mitochondrial membrane with its ethylene formation activity is exposed to the medium. The small enhancement still obtained with submitochondrial particles (fig.1) can be attributed to a small fraction of right side-out particles. In the presence of nigericin at concentra-

Table 2

Ethylene formation by intact mitochondria and by submitochondrial particles

| Fraction | Ethylene formation (nmol · mg protein$^{-1}$ · h$^{-1}$) |
|---|---|
| Intact mitochondria | 3.4 |
| Submitochondrial particles | 8.4 |
| Supernatant | 0 |
| Submitochondrial particles + supernatant | 8.0 |
| Intact mitochondria heated for 10 min at 100°C | n. d. |
| Submitochondrial particles heated for 10 min at 100°C | n. d. |

Intact mitochondria (45 mg protein/ml) and sonicated particles were prepared as in section 2. The supernatant which contains the submitochondrial particles was spun down at 100000 × *g* for 90 min. The supernatant was saved and half of the submitochondrial pellet was suspended in the homogenization buffer and brought to 4.5 mg/ml. The other half was resuspended in the supernatant (supernatant + submitochondrial particles) and brought to the same protein concentration. Assay of ethylene formation was carried out as in section 2; n.d., not detectable

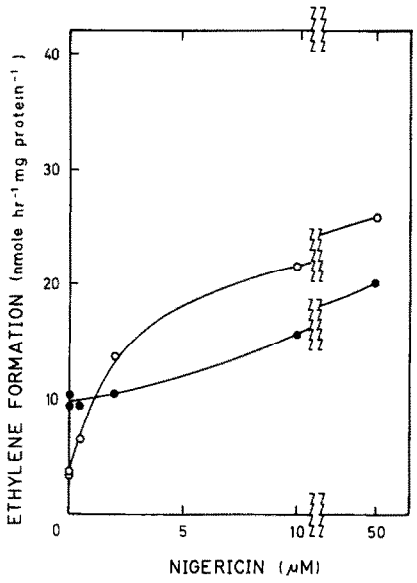


Fig.1. Effect of nigericin and $K^+$ on the rate of ACC conversion to ethylene in intact mitochondria and in submitochondrial particles; 1.0 ml of the reaction mixture contained 10 mM KCl, 0.1 mM $MnCl_2$, 100 mM K-HEPES at pH 8.0 and 190 μg of either mitochondrial or submitochondrial protein. Different nigericin concentrations were added after the addition of the membranes. The reaction was started by the addition of 10 mM ACC and ethylene formation was assayed at 20-min intervals, as described in section 2: (○) intact mitochondria; (●) Submitochondrial particles.

tions which induce maximal ethylene production, intact mitochondria produce ethylene at a slightly higher rate than sonicated submitochondrial particles. This can be attributed to a partial inhibition of the ethylene-formation system by the sonication procedure.

Ethylene formation from ACC in a cell-free system has been demonstrated in [11], [17] and [18]. The two last reports deal with a soluble system that might reflect released membrane-bound activity and its original localization is not known. The system reported in [11] is a microsomal fraction isolated from senescing carnation petals. The nature of this system is different from the one reported here and its activity is lower. The possibility that the mitochondria are involved in ethylene formation is of interest in view of the changes in the respiration rates which are so often associated with the increase in ethylene formation in the different tissues. In as much as this paper

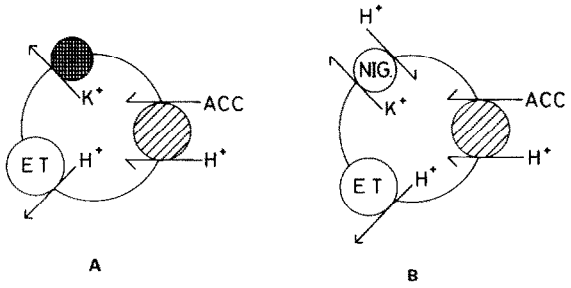


Fig.2. Diagrammatic illustration of ACC transport into the mitochondrial membrane: (A) endogenous transport mechanism; (B) transport supported by the ionophore nigericin in the presence of 10 mM KCl; NIG, nigericin; ET, electron transport; for details, see text.

suggests that the mitochondrion is a site of ethylene formation, the enzymic mechanism of its formation and the nature of the proteins involved are still an open question.

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