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Inactivation of Stress Induced 1-Aminocyclopropane Carboxylate Synthase *in Vivo* Differs from Substrate-Dependent Inactivation *in Vitro*[1]

Pietro Spanu*, Georg Felix, and Thomas Boller

Botanisches Institut der Universität Basel, Hebelstrasse 1, CH-4056 Basel, Switzerland (P.S., T.B.), and Friedrich-Miescher-Institut, P.O. Box 2543, CH-4002 Basel, Switzerland (G.F., T.B.)

ABSTRACT

The activity of 1-aminocyclopropane carboxylate (ACC) synthase increased rapidly in tomato (*Lycopersicon esculentum* Mill.) leaf discs after vacuum infiltration, reached a maximum after about 30 minutes, and subsequently decayed with an apparent half-life of about 20 minutes. Aminoethoxyvinylglycine, a known inhibitor of ACC synthase, did not alter the apparent turnover of ACC synthase *in vivo* although it efficiently blocked inactivation of the enzyme by its substrate S-adenosylmethionine *in vitro*. Similar results were obtained, using a novel assay with permeabilized cells, for ACC synthase in tomato cell cultures treated with a fungal elicitor. The results indicate that inactivation of ACC synthase *in vivo* differs from substrate-dependent inactivation *in vitro*.

Ethylene biosynthesis in higher plants involves a two-step reaction: AdoMet[2] is converted to ACC which is in turn oxidized to ethylene (14). The enzyme that catalyses the first step, ACC-S, is the main regulatory factor because induction of ethylene production is usually correlated with an increase in ACC-S activity. Density labelling experiments with D_2O showed that ACC-S was synthesized *de novo* in wounded tomato fruit (1) and in elicitor-treated parsley cell cultures (5). The enzyme appears to turn over rapidly. In wounded tomato pericarp, for example, ACC-S activity was found to decay with a half-life of 40 min in the presence of cycloheximide (7). Thus endogenous levels of ACC-S activity are determined both by the rates of enzyme synthesis and enzyme inactivation.

ACC-S is inactivated *in vitro* by its substrate, AdoMet (3, 10). A series of elegant experiments (11, 12) have shown that this process is due to irreversible covalent linkage of the 2-aminobutyrate moiety of AdoMet to the enzyme, that the (+)-AdoMet diastereoisomer has a greater inactivating potential than the (−)-AdoMet isomer and that AVG, a well-known inhibitor of ACC synthase, suppresses substrate-dependent enzyme inactivation. AVG has been found to increase ACC-S activity in mung-bean hypocotyls (15) both by uncoupling a feedback mechanism of catabolite repression and by increasing enzyme half-life *in vivo* (16). This has led to the suggestion that substrate inactivation is the cause of the rapid decrease in enzyme activity observed *in vivo* (10).

We now report that AVG does not alter the apparent half-life of stress-induced ACC-S in tomato leaf tissue and cell suspension cultures, indicating that substrate-dependent inactivation is not the cause for ACC-S decay in these systems.

MATERIALS AND METHODS

Plant Material and Treatments

Tomato seeds (*Lycopersicon esculentum* Mill., cv "Super Marmande") were sown in moist peat. Discs of 10 mm diameter were punched out leaflets from 5-week-old plants and floated on water at room temperature for up to 1 h. They were infiltrated under vacuum with water or with test solutions and frozen in liquid N_2 at the appropriate time. Tomato cells (line Msk8 (8), a generous gift of Dr. M. Koornneef, Wageningen) were grown in a Murashige-Skoog-type liquid medium supplemented with vitamins according to Adams and Townsend (2) and with 5 μM NAA and 1 μM BA. To induce ACC-S, they were treated with 10 μg mL^{-1} of a nondialysable, heat stable elicitor preparation partially purified from yeast extract (C Basse, T Boller, unpublished data).

Assays with Leaf Disc Extracts

Frozen leaf discs were extracted in 0.1 M Hepes (K^+) buffer (pH 8.0), 1 mM DTT, 0.4 μM pyridoxal phosphate (extraction buffer) and assayed for ACC content and ACC-S activity as previously described (13). ACC contents and enzyme activities were expressed per protein, determined in duplicate assays according to Bradford (4). To test the effect of AdoMet and AVG on the stability of the enzyme, crude extracts were incubated in extraction buffer containing 50 μM AVG and/or 200 μM AdoMet. After appropriate incubation times, pyridoxal phosphate (final concentration, 25 mM) was added. After an additional incubation for 15 min, the preparations were passed over a Sephadex G-50 column that was equilibrated with 2 mM Hepes (K^+) buffer (pH 8), 0.1 mM DTT, 0.2 μM pyridoxal phosphate (column buffer) and were assayed for ACC-S activity and protein as above.

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[2] Abbreviations: AdoMet, S-adenosyl methionine; ACC, 1-aminocyclopropane-1-carboxylic acid; ACC-S, 1-aminocyclopropane carboxylate synthase: AVG, aminoethoxyvinyl glycine.

Assays with Cell Cultures

To assay suspension cultures, a technique using permeabilized cells was developed; this enabled us to easily remove endogenous ACC and therefore to process up to 100 samples in a few hours. Samples of cells (2 mL, corresponding to about 0.4 g) were harvested, suspended in 2.5 mL 30 mM Hepes (K^+) buffer (pH 8.0), containing 1 mM DTT, 1% (w/v) Triton-X-100, 0.5 μM pyridoxal phosphate (permeabilizing buffer) and immediately frozen by immersing the tubes in dry ice/ethanol. After thawing at room temperature, the samples were loaded into empty disposable chromatography columns (Biorad, catalog No. 731-1550) and washed three times at room temperature with 10 packed-cell-volumes of permeabilizing buffer and then three times with the same buffer without Triton-X-100 (assay buffer). The cells were resuspended in 2 mL assay buffer. Two 1-mL portions of each sample were pipetted into test tubes. One portion received 50 μL 1 mM AdoMet (enzyme assay), the other 50 μL water (enzyme blank). The mixtures were incubated for 1.5 h at 30°C. The reaction was stopped by adding 100 μL 10 mM $HgCl_2$, and the ACC produced was measured after conversion to ethylene according to Lizada and Yang (9). Identification and quantification was carried out by comparison with the retention time and peak area of standard samples. Enzyme activities were expressed per fresh weight of the cells. To determine the stability of ACC synthase in the presence or absence of AVG or AdoMet, the permeabilized cells were incubated in assay buffer with 50 μM AVG and/or 200 μM AdoMet for appropriate times and then washed extensively (4–5 portions of 10 packed-cell-volumes) with assay buffer. Thereafter, ACC-S was determined as described above.

RESULTS

Tomato leaf tissue had a low ACC-S activity that remained at its basal level in discs punched out and floated on water. However, after vacuum infiltration of tomato leaf discs with water, ACC-S activity increased rapidly during 30 min and then decreased to background levels (Fig. 1A). Between 30 and 70 min, the decay of activity followed first-order kinetics, as indicated by the linear decay curve in a semilogarithmic plot of the data (inset in Fig. 1A). The apparent half-life during this time was determined as 21 min in the experiment shown. Values in two other cases were 14 and 30 min (not shown). Presence of AVG in the infiltrating solution did not affect either the course of induction or of decay. The ACC content of leaf discs infiltrated with water increased concomitantly with the increase in ACC-S (Fig. 1B). In leaf discs infiltrated with AVG, ACC did not accumulate in spite of the increase in enzymatic activity.

Measurements were carried out to test whether ACC-S from leaf tissue was subject to substrate-dependent inactivation *in vitro*. Crude leaf extracts were incubated at 30°C in the absence or presence of AdoMet and AVG (Fig. 2). At the times indicated, pyridoxal phosphate (final concentration 25 mM) was added. This was necessary for recovery of enzyme activity from the AVG-treated mixtures. After an additional incubation of 15 min, the homogenates were passed over a Sephadex

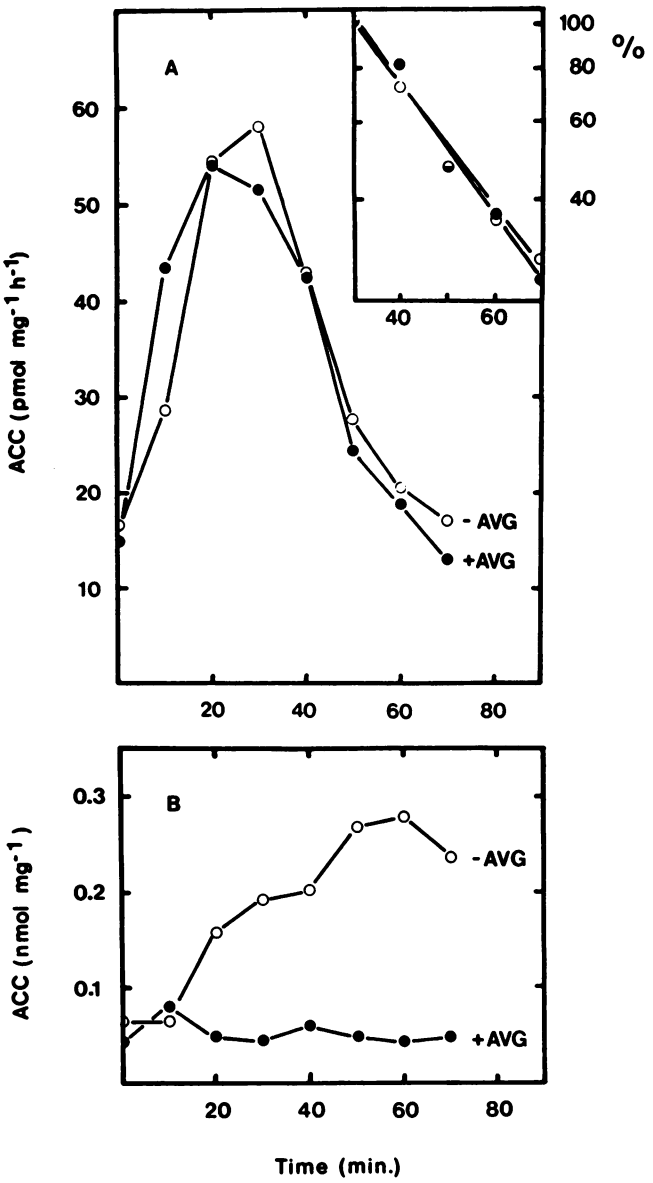

Figure 1. Changes of ACC Synthase activity (A) and of ACC levels (B) in tomato leaf discs infiltrated with water (○) or with 50 μM AVG (●). A, Specific activity of ACC-S in leaf homogenates. Inset, Values of activity during the phase of decay (30 to 70 min after infiltration) plotted semilogarithmically (activity at 30 min taken as 100%). B, ACC level in crude leaf homogenates, expressed per mg protein.

G-50 column equilibrated with column buffer and were assayed for ACC-S. In the absence of substrate, the enzyme was stable for at least 2 h. In the presence of 200 μM AdoMet, ACC-S activity decreased with first-order kinetics, displaying a half-life of 66 min. Addition of 50 μM AVG fully protected ACC-S from AdoMet-dependent inactivation.

Addition of an elicitor to cultured tomato cells (Fig. 3) caused an increase in ACC-S activity which reached a maximum at around 3.5 h and then decreased. Cycloheximide, added 2 h after the elicitor, immediately stopped any further increase of ACC-S activity. After addition of cycloheximide,

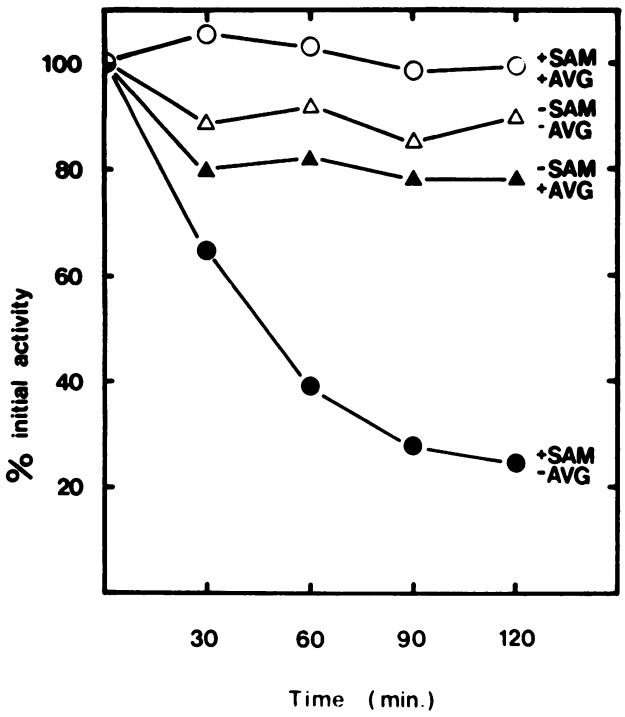

Figure 2. Decay of ACC Synthase in crude extracts from leaf discs *in vitro*. Leaf discs were infiltrated with water for 30 min and then extracted. The extract was incubated up to 120 min at 30°C in the presence of 50 μM AVG and/or 200 μM AdoMet. At the time indicated, pyridoxal phosphate was added, the samples were passed over Sephadex G-50 columns, and the specific activity of ACC-S was measured. Values represent percentage of the initial specific activity (80 pmol per mg protein per h).

ACC-S activity decayed with an apparent half-life of 40 min (Fig. 3). The same half-life was found when cycloheximide was given after 4 h (data not shown). Addition of 50 μM AVG together with the cycloheximide did not alter the decay of ACC-S activity (Fig. 3). In another experiment with a different batch of cells, the apparent half-life was found to be 50 min after addition of cycloheximide alone and 60 min after addition of cycloheximide and AVG (data not shown).

In permeabilized cells, activity of ACC-S remained stable for at least 2 h in the absence of AdoMet and AVG (Fig. 4). In the presence of 200 μM AdoMet, the activity of ACC-S decayed with approximate first-order kinetics, showing an apparent half-life of 67 min. Addition of 50 μM AVG together with the AdoMet prevented this substrate-dependent inactivation.

DISCUSSION

Induction of ethylene biosynthesis is a rapid response of plant tissues to a variety of stressful conditions (14). This is confirmed by our investigations, which show a rapid transient increase in ACC-S activity in leaf discs after vacuum infiltration with water and in cell cultures after treatment with elicitor. ACC-S is also subject to rapid inactivation, with apparent half-lives in leaves and cell cultures *in vivo* of about 20 and 40 min, respectively. These values are similar to those already determined for green tomato pericarp (7) and auxin-treated mung bean hypocotyls (16). In cell cultures, the half-life is similar 2 h and 4 h after addition of elicitor, indicating that the elicitor-induced increase in ACC-S is not due to a decrease in its rate of decay.

In leaf homogenates and in permeabilized, washed cells ACC-S does not show any turnover; in both cases, the activity is stable for at least 2 h at 30°C. However, addition of AdoMet results in a time-dependent inactivation of ACC-S with an apparent half life of about 1 h, a value in close agreement with that reported for tomato pericarp derived enzyme *in vitro* (11). In the latter case, inactivation has been found to be caused by irreversible covalent binding of a AdoMet fragment to the enzyme in what appears to be a side reaction of the interaction of AdoMet with the catalytic site of the enzyme (11). In line with this is the finding that AVG, a pyridoxal phosphate binding inhibitor, efficiently protects ACC-S from substrate-dependent inactivation (3, 10–12). In our experiments, we have demonstrated a similar protection of ACC-S *in vitro*, both in leaf homogenates and permeabilized cells. In the case of leaf homogenates, we initially had been troubled by the finding that AVG appeared to inactivate the enzyme too. However, we then found that the full activity of ACC-S can be recovered from AVG-treated leaf homogenates by an incubation with 25 mM pyridoxal phosphate for 15 min, a

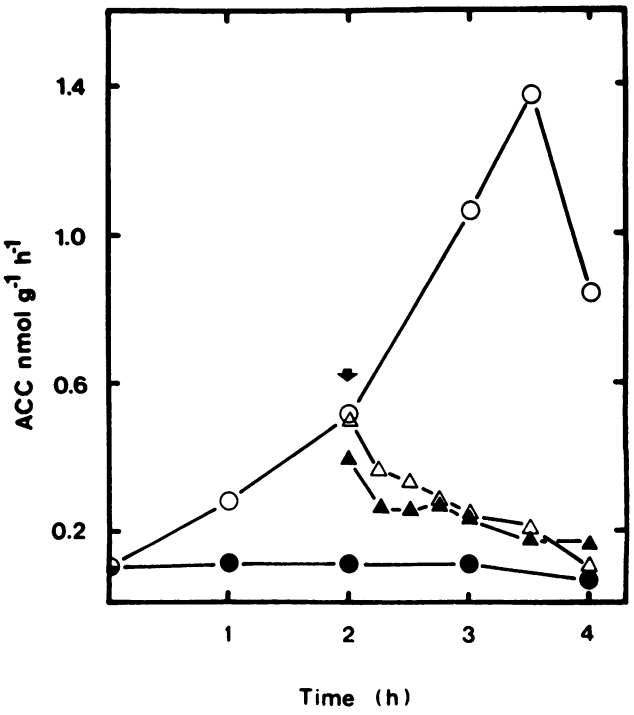

Figure 3. Changes of ACC synthase activity in suspension-cultured tomato cells without additions (●) or after a treatment with 10 μg mL^{-1} elicitor (○) *in vivo*. To parts of the elicitor-treated cells, 20 μM cycloheximide (△) or 20 μM cycloheximide and 50 μM AVG (▲) were added after 2 h (arrow). ACC-S activity (nmol per g fresh weight per h) was determined in permeabilized cells.

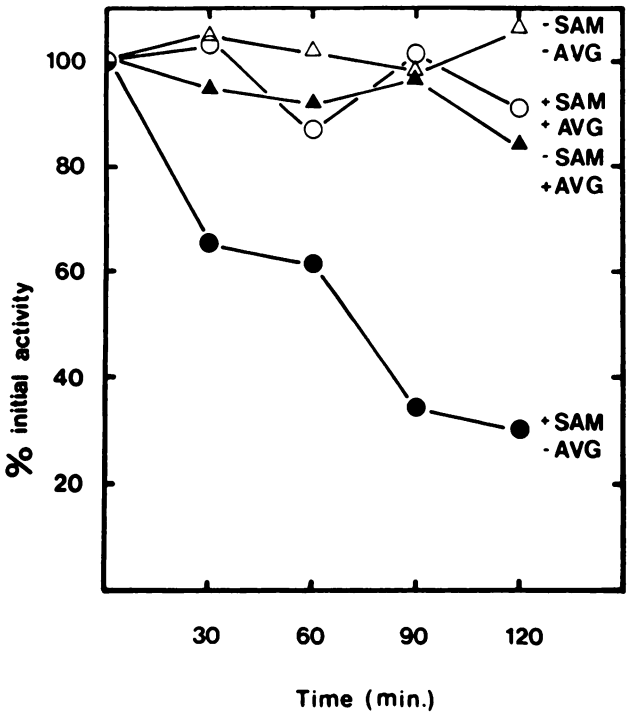

Figure 4. Decay of ACC synthase activity in permeabilized suspension-cultured tomato cells *in vitro*. Cells were permeabilized after incubation with 10 μg mL^{-1} elicitor for 3 h. The permeabilized cells were incubated in assay buffer without further additions or with 200 μM AdoMet and/or 50 μM AVG. After the times indicated, they were washed extensively with assay buffer supplemented with 5 μM pyridoxal phosphate and then assayed for ACC-S. Values represent percentage of the initial activity (1.3 nmol per g fresh weight per h).

treatment recommended for β-cystathionase, another pyridoxal phosphate-dependent enzyme, after inhibition by rhizobitoxin, an analogue of AVG (6).

In contrast to the situation *in vitro*, addition of AVG to leaf tissue and cell cultures does not alter ACC-S turnover *in vivo* (Fig. 1A and Fig. 2). The fact that AVG blocks the increase in ACC levels in the leaf discs (Fig. 1B) clearly indicates that it has access to the catalytic site of ACC-S. Therefore, AVG is expected to block any possible substrate-dependent inactivation too. Similar experiments indicate that AVG also blocks catalytic activity in cultured cells (results not shown). Our results clearly show that there is no reduction of the decay of ACC-S in the presence of AVG. This leads to the conclusion that the rapid turnover of ACC-S *in vivo* is not due to substrate-dependent inactivation, at least not in leaf tissue or cultured cells of tomato. Further investigations will be needed to determine the mechanisms of rapid enzyme turnover *in vivo* which is of great importance in the regulation of ACC-S levels and ultimately of ethylene biosynthesis in higher plants.

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