Conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene in submitochondrial particles isolated from plants

Characterization of the system

Chana Vinkler and Akiva Apelbaum*

Department of Fruit and Vegetable Storage, Agricultural Research Organization, The Volcani Center, PO Box 6, Bet Dagan 50250, Israel

Received 6 December 1983

Conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene was studied in submitochondrial particles (SMP) which were prepared by sonication of the mitochondrial fraction isolated from 7-day-old etiolated pea (*Pisum sativum* var. Kelvedon Wonder) seedlings. The reaction was enhanced by the addition of Mn^{2+} and had a maximal rate at pH 8.0. Conversion of ACC to ethylene was inhibited under anaerobic conditions and by the addition of KCN, EDTA, NaN_3, *n*-propyl gallate and $CoCl_2$. Addition of the uncouplers 2,4-DNP, CCCP and FCCP, however, did not inhibit the reaction. Structural analogs of ACC inhibited ACC conversion to ethylene by SMP. The structural analog of methionine, α-keto-γ-methylthiobutyric acid (KMB), was converted to ethylene by SMP at a rate which was only about 2% that of ACC conversion to ethylene.

Ethylene *1-Aminocyclopropane-1-carboxylic acid* *Submitochondrial particle* *Inhibitor* *Substrate analog*

1. INTRODUCTION

In a recent study we demonstrated the formation of ethylene from its precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), in the mitochondrial fraction isolated from etiolated pea seedlings [1]. A 2- to 3-fold increase in the rate of this reaction was obtained with sonicated submitochondrial particles (SMP) over the rate measured with intact mitochondria. Since SMP are inside-out vesicles of the inner mitochondrial membrane [2,3], it was concluded that biosynthesis of ethylene from ACC in plant mitochondria is associated with the inner mitochondrial membrane.

Ethylene formation in plant organs or in plant tissues has been largely characterized by the use of specific inhibitors of ethylene formation, structural analogs of the substrates of the reaction, and various treatments of the ethylene-forming tissue which affected the reaction. The identification of intermediates in the biosynthetic pathway leading to ethylene formation [4–6] made it possible to gain more information about the site of inhibition of some of the inhibitors, the nature of the reaction it inhibited and the factors involved in it.

Aminoethoxy-vinylglycine (AVG) and aminooxyacetic acid (AOA), which are known to be inhibitors of pyridoxal enzymes, inhibit the conver-

* To whom correspondence should be addressed

Abbreviations: SMP, submitochondrial particles; ACC, 1-aminocyclopropane-1-carboxylic acid; 2,4 DNP, 2,4-dinitrophenol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; KMB, α-keto-γ-methylthiobutyric acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

sion of *S*-adenosylmethionine (SAM) to ACC [7,8]. This step precedes the final step of ethylene biosynthesis, namely, ACC conversion to ethylene. Cobalt ion strongly inhibits ethylene production, both in apple tissues and in mung bean hypocotyls [9]. Co^{2+} was found to promote many growth processes which are inhibited by ethylene and which were shown to inhibit ethylene formation in mung bean hypocotyls while, at the same time, ACC was accumulated [10]. It has therefore been suggested that Co^{2+} inhibits the final step of ACC conversion to ethylene. Ethylene synthesis was also inhibited under anaerobic conditions. The conversion of ACC to ethylene [11] was considered the O_2-dependent step of this reaction. This step was also considered as the site of inhibition of the uncouplers 2,4-dinitrophenol and CCCP [12,13].

Structural analogs of ACC [13] and of other intermediates in the reaction [14,15] were added to the ethylene-forming system in apple slices, cauliflower and tomato tissue. Of these analogs, both methional and α-keto-γ-methylthiobutyric acid (KMB), which are structural analogs of methionine, may be degraded to ethylene under certain conditions [16,17]. The early suggestions that they are intermediates in the pathway of ethylene biosynthesis were contradicted when differences in the nature of labeled by-products were found between the system which uses labeled methionine and the one utilizing labeled KMB [18].

Here we used SMP in an attempt to characterize and compare the ethylene-forming system in the cell-free preparation, to the one in the intact tissue. The use of SMP was preferred over the use of intact mitochondria, since the former seems to have the ethylene-forming system in an orientation which is exposed to the medium and hence it was not necessary to consider transport of ACC across the membrane as a kinetic limitation.

ACC conversion to ethylene by submitochondrial particles was found to be specific to ACC as a substrate and was abolished when inhibitors of the reaction in vivo were added to the assay medium.

2. MATERIALS AND METHODS

Plumular hooks from 7-day-old etiolated pea seedlings (*Pisum sativum* var. Kelvedon Wonder) were excised and homogenized, the mitochondrial fraction was isolated, and submitochondrial particles were prepared as in [1].

The conversion of ACC to ethylene was assayed in 1.0 ml of reaction mixture which, unless otherwise mentioned, contained: 100 mM Na–Hepes buffer at pH 8.0, 0.1 mM $MnCl_2$, 10 mM ACC and submitochondrial particles (at a final concentration of 0.2–0.4 mg protein/ml). The reaction mixture was placed in a test tube, closed with a rubber septum immediately after the addition of the substrate, and placed in a shaking water bath at 28°C. Ethylene production was measured as in [1], at 20-min intervals. Ethylene formation was linear with time for at least 1 h. Negligible amounts of ethylene were obtained with heat-denatured control samples.

3. RESULTS AND DISCUSSION

3.1. *Effect of pH and of divalent cations on ACC conversion to ethylene by submitochondrial particles*

Fig.1 depicts the dependence of the rate of ethylene formation by SMP on the pH of the medium. A maximal value was obtained at pH 8.0. This activity was inhibited by more than 90% when either Tris or Tricine buffer was used instead of Hepes buffer at the same pH. Since Tricine is

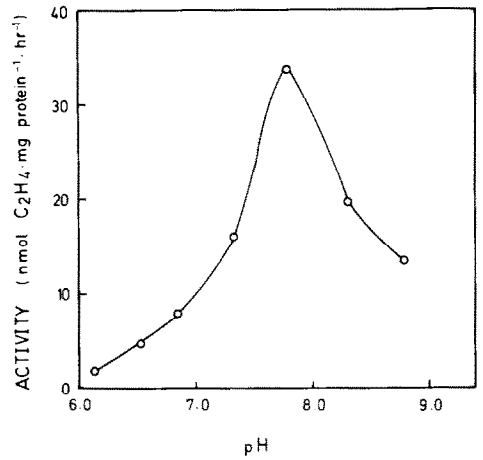

Fig.1. Effect of pH on ACC conversion to ethylene. The reaction mixture (1.0 ml) contained 100 mM buffer at the indicated pH, 10 mM KCl, 0.1 mM $MnCl_2$, 10 mM ACC and 0.24 mg SMP. Pipes buffer was used between pH 6.0 and 7.0, Hepes buffer was used between pH 7.0 and 8.0 and CAPS buffer was used between pH 8.0 and 9.0

known to bind divalent cations [19], its inhibitory effect could be due to the removal of divalent cations, which are necessary in the reaction. Table 1 describes the effect of various divalent cations on ethylene formation by SMP, $MnCl_2$ caused a more than 20-fold increase in the rate of ACC conversion to ethylene over the control, whereas $CaCl_2$, $MgCl_2$ or $CuCl_2$ had a relatively small effect on this reaction. It has been reported that ethylene formation from ACC by a soluble fraction isolated from etiolated pea seedlings, is greatly enhanced by the addition of $MnCl_2$ to the reaction mixture [20]. The mitochondrial ethylene-formation activity reported here is similar to the previously reported soluble system not only in its dependence on $MnCl_2$ and in the concentration of $MnCl_2$ which yields maximal activation (fig.2), but also in the pH value obtained for optimal ethylene-formation activity [21].

3.2. *Effect of uncouplers and of different inhibitors on the conversion of ACC to ethylene by submitochondrial particles*

Uncouplers of oxidative phosphorylation were shown to inhibit ethylene formation in ethylene-producing tissues at concentrations which cause complete uncoupling in isolated mitochondria. Addition of 0.1 mM 2,4-dinitrophenol to mung bean hypocotyls caused about 85% inhibition of both ACC- and IAA-dependent ethylene formation [12]. The uncoupler, CCCP, inhibited ethylene formation in the same system at a much lower concentration (I_{50} = 5 μM). Similar results were obtained in apple slices and in etiolated pea seedlings [13], where a significant reduction in ATP concentration was demonstrated concomitantly. As seen in table 2, CCCP, FCCP and 2,4 DNP caused only a slight inhibition of ethylene production by SMP under similar conditions. Similar results were obtained with intact mitochondria (not shown). The reason for the different responses of the isolated mitochondrial system and the ethylene-forming tissue to the addition of uncouplers is still obscure. One possible explanation may be that ACC conversion to ethylene in vivo involves an ATP-dependent step which precedes the conversion reaction itself, either directly or indirectly (e.g., the transport of ACC in vivo to the intracellular site of conversion).

As mentioned above, metal chelators inhibit ethylene formation in SMP. The inhibitory effect of EDTA, as well as other metal chelators, has already been demonstrated in apple tissues [4]. In SMP, ethylene formation is inhibited by EDTA (table 2), and this inhibition can be reversed when the concentration of Mn^{2+} in the assay medium is elevated (not shown). More information on the

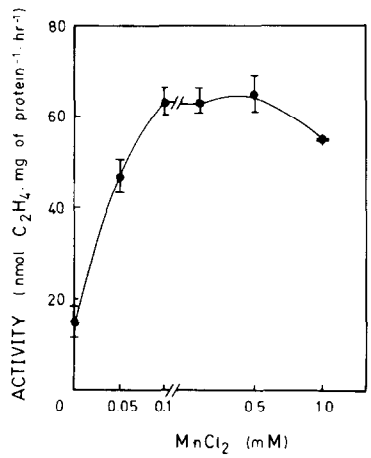

Fig.2. Effect of $MnCl_2$ concentration on the conversion of ACC to ethylene. Reaction mixture was as described in section 2. $MnCl_2$ was added at variable concentrations as indicated in the figure. SMP was added at a final protein concentration of 0.4 mg/ml.

Table 1

The effect of divalent cations on ethylene formation from 1-aminocyclopropane-1-carboxylic acid in submitochondrial particles

Addition	Cation concentration (mM)	Ethylene formation (nmol · mg protein^{-1} · h^{-1})
–	–	1.15
$MgCl_2$	1.0	1.60
	5.0	1.62
$CaCl_2$	1.0	2.14
$CuCl_2$	1.0	2.55
$MnCl_2$	0.1	31.66
	1.0	20.00

Ethylene formation was determined in a 1.0-ml reaction mixture containing 100 mM Na–Hepes at pH 8.0, 10 mM KCl, 10 mM ACC, 25 μl submitochondrial particles (15.5 mg protein/ml) and various divalent cations, as indicated, at 28°C

Table 2

Effect of uncouplers and of various inhibitors on ACC conversion to ethylene in submitochondrial particles

Addition	Concentration	Ethylene formation (nmol·mg protein^{-1}·h^{-1})	% of control
–	–	8.22	100
Anaerobiosis	–	1.86	22
FCCP	10 μM	5.76	70
CCCP	10 μM	6.92	84
DNP	5 mM	6.15	75
EDTA	0.5 mM	u.d.	–
KCN	0.1 mM	2.76	33
	1.0 mM	0.88	11
$CoCl_2$	5 μM	4.27	52
	50 μM	1.07	13
NaN_3	1 mM	1.36	16
n-Propyl gallate	5 μM	2.05	25
	20 μM	0.33	4

The assay medium contained 100 mM Na–Hepes buffer at pH 8.0, 10 mM KCl, 0.1 mM $MnCl_2$, 10 mM ACC and submitochondrial particles (0.304 mg protein) in a final volume of 1.0 ml. The test tubes were capped and placed in a water bath at 28°C. Samples from the gaseous phase were withdrawn at intervals of 20 min for analysis of ethylene concentration; u.d., undetectable

Table 3

Ethylene formation by submitochondrial particles in the presence of various substrates

Addition	Ethylene formation (nmol·mg protein^{-1}·h^{-1})	% of control
10 mM ACC	8.22	100
10 mM cyclopropane carboxylic acid	u.d.	–
10 mM ACC + 10 mM cyclopropane carboxylic acid	2.04	25
10 mM cyclopropyl-methylcarbinol	u.d.	–
10 mM ACC + 10 mM cyclopropylmethyl-carbinol	5.92	72
10 mM KMB	0.19	2
10 mM ACC + 10 mM KMB	11.04	134

Submitochondrial particles were added to 1.0 ml medium as described in table 2 at a final protein concentration of 0.304 mg/ml. Unless otherwise indicated, ACC was omitted from the assay medium. Measurements of ethylene formation were performed as described in section 2; u.d., undetectable

nature of the ethylene-forming enzyme is needed in order to understand the role of Mn^{2+} in the mechanism of the reaction.

KCN, *n*-propyl gallate and $CoCl_2$, which are known to inhibit ethylene production in ethylene-forming tissue [4,9,10,13], were effective even at lower concentrations in isolated submitochondrial particles (table 2). The mode of action of these inhibitors in the ethylene-formation reaction is not known; however, *n*-propyl gallate is a free radical scavenger and it has been thought for a long time that its inhibitory effect on ethylene formation in tissues indicates that free radicals are involved in the conversion of ACC to ethylene [22]. It should be noted that *n*-propyl gallate has recently been shown [23] to inhibit the mitochondrial alternate electron transport pathway at concentrations similar to those which inhibit ethylene formation in SMP (table 2). KCN is a very well known inhibitor of cytochrome oxidase, the enzyme which carries out the final step in the chain of oxidation reactions in the mitochondria. The sensitivity of the reaction to anaerobic conditions, together with the inhibitory effect of KCN on the reaction, are in line with the suggestion made about a decade ago that cytochrome oxidase may be involved in the reaction [11].

3.3. *The effect of structural analogs of ACC and of KMB on ethylene formation in submitochondrial particles*

Table 3 shows the effect of two analogs of ACC, cyclopropane carboxylic acid and cyclopropylmethylcarbinol, on ethylene formation in SMP. Both analogs inhibited ethylene formation from ACC, but their mode of inhibition is still under investigation. These compounds did not inhibit ethylene formation in either apple cortex or bean leaf [13]. This may be due to the slow penetration of these substrates into the cytoplasm of the cell.

SMP converts KMB to ethylene at a rate of about 2% that of the conversion of equimolar concentrations of ACC to ethylene (table 3). In contrast, conversion of KMB to ethylene by homogenates of etiolated pea seedlings ([21], fig.2b) was reported to occur at a rate similar to that of ACC conversion to ethylene under the same conditions. Whereas very slow degradation of KMB to ethylene was observed when the former was added to SMP (table 3), it caused a synergistic enhancement of ACC conversion to ethylene in this system. The reason for the activation effect is still obscure. Degradation of KMB to ethylene was previously attributed to oxidases present in many of the ethylene-forming tissues [23]. It has also been demonstrated in a model system developed in [24], in which horseradish peroxidase, sulfite, specific phenols and manganese or catalytic amounts of H_2O_2 converted methional or KMB to ethylene.

We may thus conclude that SMP converts ACC to ethylene in a mode which is rather specific to ACC and is similar both in its characteristics and its sensitivity to inhibitors, to the system described in vivo.

ACKNOWLEDGEMENTS

This investigation was supported by the US-Israel Binational Agricultural Research and Development Fund (BARD), no.I-221-80. We thank Mrs H. Heller and Mrs S. Abramowitz for their excellent technical help in performing this research. This is contribution no.924-E, 1983 series, from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel.

REFERENCES

[1] Vinkler, C. and Apelbaum, A. (1983) FEBS Lett. 162, 252–256.
[2] Lee, C.P. and Ernster, L. (1966) Proc. Symp. 1965 BBA Libr. 7, 218–234.
[3] Mitchell, P. (1966) Biol. Rev. 41, 445–502.
[4] Lieberman, M., Kunishi, A.T., Mapson, L.W. and Wardale, D.A. (1966) Plant Physiol. 41, 316–382.
[5] Adams, D.O. and Yang, S.F. (1977) Plant Physiol. 60, 892–896.
[6] Adams, D.O. and Yang, S.F. (1979) Proc. Natl. Acad. Sci. USA 76, 170–174.
[7] Boller, T., Herner, R.C. and Kende, H. (1979) Planta 145, 293–303.
[8] Yang, S.F. (1980) Hort. Sci. 15, 238–243.
[9] Lau, O.L. and Yang, S.F. (1976) Plant Physiol. 58, 114–117.
[10] Yu, Y.B. and Yang, S.F. (1979) Plant Physiol. 64, 1074–1077.
[11] Burg, S.P. (1973) Proc. Natl. Acad. Sci. USA 70, 591–597.
[12] Yu, Y.B., Adams, D.O. and Yang, S.F. (1980) Plant Physiol. 66, 286–290.
[13] Apelbaum, A., Wang, S.Y., Burgoon, A.C., Baker, J.E. and Lieberman, M. (1981) Plant Physiol. 67, 74–79.
[14] Mapson, L.W., March, J.F. and Wardale, D.A. (1969) Biochem. J. 102, 574–585.
[15] Mapson, L.W. and Wardale, D.A. (1968) Biochem. J. 107, 433–442.
[16] Ku, H.S., Yang, S.F. and Pratt, H.K. (1969) Phytochemistry 8, 567–573.
[17] Demorest, D.M. and Stahmann, M.A. (1971) Plant Physiol. 47, 450–451.
[18] Lieberman, M. and Kunishi, A.T. (1971) Plant Physiol. 47, 576–580.
[19] Good, N. and Izawa, S. (1972) in: Methods in Enzymology (San Pietro, A. ed) vol.24, pp.53–86, Academic Press, London, New York.
[20] Konze, J.R. and Kwiatkowsky, G.M.K. (1981) Planta 151, 320–326.
[21] Konze, J.R. and Kende, H. (1979) Planta 146, 293–301.
[22] Baker, J.E., Lieberman, M. and Anderson, J.D. (1978) Plant Physiol. 61, 886–888.
[23] Siedow, J.N. and Girvin, M.E. (1980) Plant Physiol. 65, 669–674.
[24] Mapson, L.W. (1970) Endeavor 39, 29–33.
[25] Yang, S.F. (1967) Arch. Biochem. Biophys. 122, 481–487.