In Vivo and in Vitro 1-Aminocyclopropane-1-carboxylic Acid Oxidase Activity in Pear Fruit: Role of Ascorbate and Inactivation during Catalysis

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1-Aminocyclopropane-1-carboxylic acid (ACC) oxidase catalyzes the final step in the biosynthesis of ethylene. The enzyme extracted from ripe pear fruits (*Pyrus communis* L. cv. Blanquilla) uses ascorbate as an essential cosubstrate, nonreplaceable by other reductants, and is inhibited by 2-oxoglutarate and catalase. ACC oxidase has a half-life of 60 min. Inactivation of the enzyme is partly associated with low protein concentration during incubation. The inclusion of ascorbate results in additional loss of its activity, maximum loss occurring with ascorbate, Fe^{2+}, and ACC. Inactivation is not affected by the inclusion of CO_2, and it is not due to inhibition by ethylene. In vivo, activity requires ACC, Fe^{2+}, and CO_2 for maximal activity. Ascorbate appears to act as an inhibitor in vivo, despite its requirement for the in vitro assay.

Keywords: *1-Aminocyclopropane-1-carboxylic acid oxidase; ascorbate; ethylene; enzyme inactivation; pear (Pyrus communis L.)*

INTRODUCTION

Ethylene is a plant hormone that regulates many aspects of plant physiology, including fruit ripening and senescence (Yang and Hoffman, 1984; Kende, 1993). The final step of its biosynthesis is catalyzed by 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase, which converts ACC into ethylene (Adams and Yang, 1979). ACC oxidase is a member of the 2-oxoacid-dependent dioxygenase family of enzymes (Hamilton et al., 1990; Prescott, 1993). All 2-oxoacid-dependent dioxygenases are soluble enzymes that require Fe^{2+} and ascorbate for optimal substrate conversion in vitro. However, ACC oxidase is an unusual enzyme in this group as it does not use 2-oxoglutarate as a reductant; this function is apparently carried out by ascorbate (Prescott and John, 1996). ACC oxidase uses ascorbate as a cosubstrate, oxidizing it stoichiometrically to dehydroascorbate (Dong et al., 1992). Also unusual among 2-oxoacid-dependent dioxygenases, ACC oxidase requires CO_2 as an essential cofactor (Dong et al., 1992).

In vitro ACC oxidase catalysis is nonlinear, with the enzyme extracted from many plants (Pirrung et al., 1993; Moya-León and John, 1994; Nijenhuis-de Vries et al., 1994; Dupille and Zacarías, 1996a; Escribano et al., 1996), and this nonlinearity was attributed to catalytic inactivation and H_2O_2 attack on the enzyme (Smith et al., 1994).

We have previously reported that pear ACC oxidase characterized in crude extracts resembles closely ACC oxidase from other fruits (Vioque and Castellano, 1994a,b). The aims of the present work were (i) to determine the effect of different reductants, 2-oxoglutarate and catalase, on the ACC oxidase extracted from pear fruits, (ii) to investigate the factors that are involved in the in vitro enzyme inactivation, and (iii) to examine the effect of exogenous cofactors on the in vivo ACC oxidase activity.

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MATERIALS AND METHODS

Plant Material. Pear (*Pyrus communis* L. cv. Blanquilla) fruits were purchased in a local market. Ethylene production was monitored to ascertain that the fruits were at the climacteric stage. The fruits were stored at 4 °C until needed.

Enzyme Preparation. ACC oxidase was extracted according to the method of Vioque and Castellano (1994b) with slight modifications. Pericarp tissue (300 g) was cut and homogenized for 2 min with a blender in 400 mL of 400 mM potassium phosphate buffer (pH 6.5) containing 10 mM sodium bisulfite, 3 mM sodium ascorbate, 5 mM DTT, and 4 mM 2-mercaptoethanol. The homogenate was squeezed through four layers of cheesecloth, filtered through one layer of Miracloth, and centrifuged at 25000*g* for 30 min, and the supernatant was discarded. The pellet was resuspended in 20 mL of 25 mM HEPES (pH 6.7) containing 1 mM DTT, 3 mM sodium ascorbate, and 30% glycerol (v/v) and stirred for 15 min. Triton X-100 (0.8%) was added and the mixture stirred for 15 min and centrifuged at 25000*g* for 30 min. The supernatant was assayed directly, after passing it through a column of Sephadex G-25 (PD-10), or stored at −20 °C for future use. All steps were carried out at 4 °C. Protein contents were determined according to the method of Bradford (1976) using BSA as a standard.

Enzyme Activity Assays. An in vitro standard reaction mixture consisted of 100 mM HEPES (pH 6.7), 10% glycerol (v/v), 1 mM ACC, 0.2 mM $FeSO_4$, 10 mM sodium ascorbate, 16% CO_2 (in the gas phase), and 0.1 mL (0.3−0.4 mg of protein) of enzyme solution in a total volume of 1 mL. Incubation was carried out with shaking at 28 °C for 30 min, in sealed 10 mL vials. Ethylene produced and released to the gas phase was determined by gas chromatography injecting 1 mL of headspace on a HP 5890 A GC, equipped with a flame ionization detector and an activated alumina column.

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Table 1. In Vitro Pear ACC Oxidase Activity with Different Reductants

reductant	ACC oxidase activity (nmol of C_2H_4 g^{-1} h^{-1})
none	0.02 ± 0.00
10 mM ascorbate	14.85 ± 0.75
5 mM glutathione	0.04 ± 0.00
1 mM NADH	0.02 ± 0.02
1 mM NADPH	0.02 ± 0.00
5 mM DTT	0.10 ± 0.02
10 mM ascorbate + 5 mM DTT	15.20 ± 0.84

For in vivo assays, 10 pear fruit disks (~0.5 g) were incubated at 28 °C in sealed 10 mL vials in a total volume of 1 mL containing 10 mM ACC and 0.4 M mannitol. When indicated, exogenous cofactors were added at the following concentrations: 0.2 mM $FeSO_4$, 10 mM sodium ascorbate, and 16% CO_2 (in the gas phase).

All determinations were made in triplicate (in vitro) or with five replicates (in vivo), and the results are expressed as means ± SE.

Preincubations. The enzyme extract was subjected to different preincubation treatments, which were carried out for 30 min at 28 °C with shaking in the presence of 100 mM HEPES (pH 6.7) and 10% glycerol (v/v). When indicated, different additions were made at the following concentrations: 1 mM ACC, 0.2 mM $FeSO_4$, 10 mM ascorbate, 16% CO_2, and 1 mg mL^{-1} BSA. At the end of this period, the vials were opened and ventilated (if necessary), and the enzyme activity was assayed after the addition of the required substrates and cofactors to provide the complete reaction mixture.

RESULTS

In Vitro ACC Oxidase Activity. We have previously reported the in vitro dependence of pear ACC oxidase activity on ascorbate concentration, showing a K_m value of 2.87 mM (Vioque and Castellano, 1994b). To study the possible substitution of ascorbate by other reductants, ascorbate and DTT present in the enzyme preparation were removed by passing it through a column of Sephadex G-25. Pear ACC oxidase has an absolute requirement for ascorbate, whereas glutathione, NADH, NADPH, and DTT were not able to replace ascorbate as a reducing agent (Table 1). The reinforcement of the reductant environment by the addition of 5 mM DTT to a medium that contained ascorbate did not increase the level of activity. In this respect, pear ACC oxidase, as for enzyme extracted from other plants (McGarvey and Christoffersen, 1992; Smith et al., 1992), resembles the 2-oxoacid-dependent dioxygenases, where maximum activities are observed only with ascorbate (Prescott and John, 1996).

Pear ACC oxidase activity was not stimulated by the addition of 2-oxoglutarate but was inhibited at concentrations of ~1 mM (Figure 1). A similar inhibitory effect was also reported for ACC oxidase from apple (Dupille and Zacarías, 1996b) and pear (Iturriagagoitia-Bueno et al., 1996). These latter authors found that inhibition by the 2-oxoacid was competitive with respect to ascorbate, concluding that 2-oxoglutarate binds to the enzyme at the ascorbate-binding site.

Catalase addition stimulates ACC oxidase activity from other plant systems (Smith et al., 1994; Zhang et al., 1995; Escribano et al., 1996). Therefore, we assayed the effect of the addition of catalase from bovine liver (25 000 units/ per mg of protein) on pear ACC oxidase activity. Our results clearly indicate that catalase inhibits pear ACC oxidase activity, both before (data not shown) and after the enzyme preparation was passed through a column of Sephadex G-25 (Figure 2). Although catalase did not stimulate the activity of unpurified enzyme assayed in the extract from melon (Smith et al., 1992), an inhibitory effect of catalase has not yet been reported.

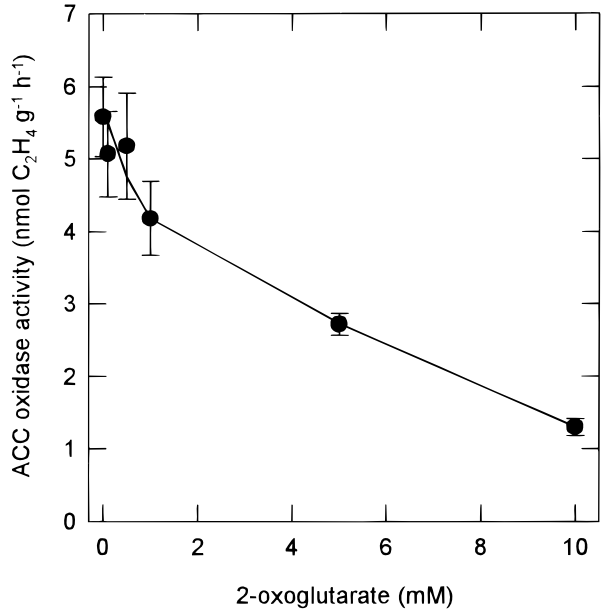

Figure 1. Effect of 2-oxoglutarate on in vitro pear ACC oxidase activity. 2-Oxoglutarate at various concentrations was included in the reaction mixture.

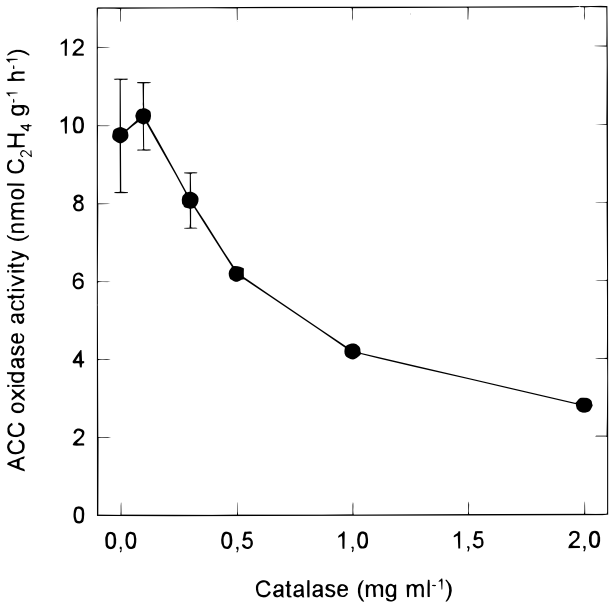

Figure 2. Effect of catalase on in vitro pear ACC oxidase activity. Catalase from bovine liver (25 000 units/mg of protein) at various concentrations was included in the assay medium.

Enzyme Inactivation. A time course during a typical in vitro assay of pear ACC oxidase shows a loss of linearity after 50 min of incubation (Vioque and Castellano, 1994a). The decline in ACC oxidase activity during incubation is not due to limitation of the substrates or cofactors during the reaction, because the ethylene production was not affected by their re-addition after a catalysis period. Figure 3 shows the residual ACC oxidase activity as a function of time. The enzyme loses activity during turnover with a half-life of 60 min. Smith et al. (1994) reported a half-life of 14 min for the purified tomato ACC oxidase, while Pirrung et al. (1993) estimated it as 2 h for the apple enzyme. The inactiva-

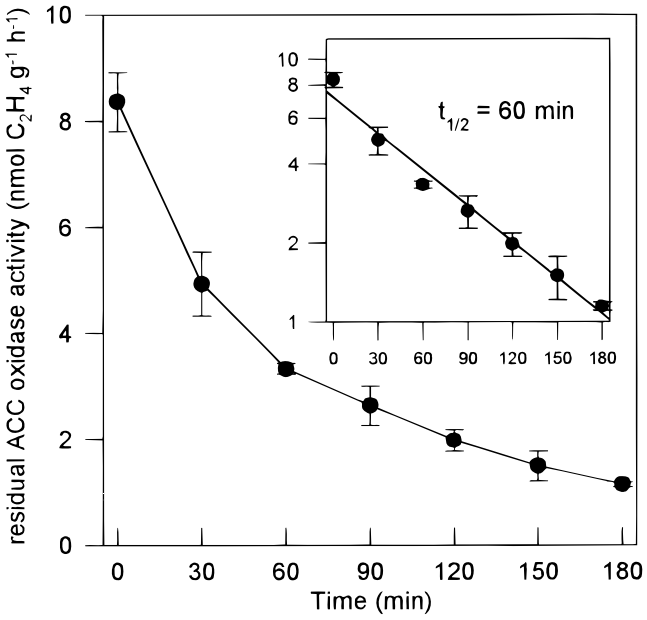

Figure 3. Time course of residual ACC oxidase activity; (inset) half-life during turnover.

Table 2. In Vitro Pear ACC Oxidase Activity after Enzyme Preincubation with Different Additions

	ACC oxidase activity	
preincubation medium	nmol of C_2H_4 g^{-1} h^{-1}	%
no preincubation	9.83 ± 0.63	100
no additions	5.70 ± 0.25	58
BSA	7.37 ± 0.88	75
BSA + ascorbate	5.01 ± 0.22	51
BSA + complete mixture	3.05 ± 0.29	31
BSA + ethylene	7.52 ± 0.19	77

tion of pear ACC oxidase is not the result of inhibition by small molecular weight compounds present in the extract, since desalting the enzyme preparation through a Sephadex G-25 column did not have any effect on the enzyme inactivation.

To investigate the causes of the inactivation, the enzyme preparation was preincubated for 30 min under different conditions and the residual activity was determined (Table 2). In these experiments we used an enzyme preparation subjected to gel filtration on a Sephadex G-25 PD-10 column, where the enzyme was eluted with resuspension buffer without ascorbate. Preincubation of pear ACC oxidase at 28 °C in buffer alone resulted in the loss of ~42% of its activity. This loss of activity was partly prevented by the addition of BSA (1 mg mL^{-1}) during the preincubation. Thus, the inactivation of ACC oxidase observed in our experiments is partly due to enzyme instability caused by incubating it at low protein concentration at 28 °C. A conformational change of fully active ACC oxidase to give an inactive or substantially less active enzyme has been pointed as the reason for this inactivating process (Barlow et al., 1997). Table 2 also shows that the inclusion of ascorbate in the preincubation medium led to an additional loss of activity (~24%) and that the inactivation was maximum when the enzyme was preincubated with the complete reaction mixture. Similar results have been reported for the ACC oxidase from melon (Smith and John, 1993) and tomato (Smith et al., 1994).

Because pear ACC oxidase undergoes inactivation during the reaction, the end product inhibition was also

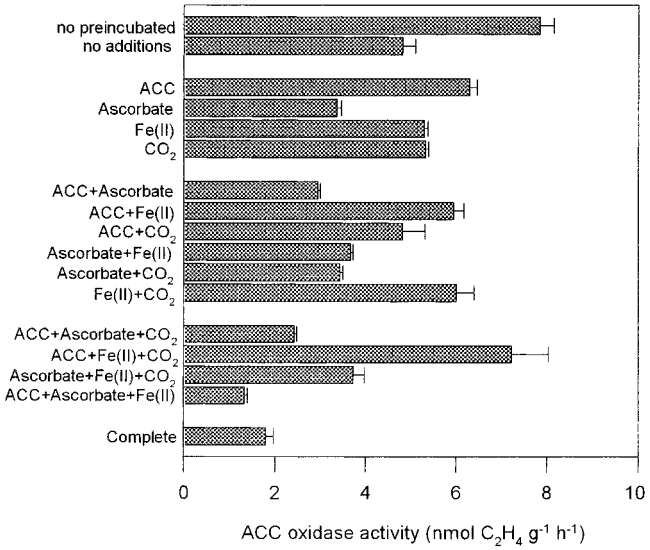

Figure 4. Effect of reaction components during preincubation on in vitro pear ACC oxidase activity. The enzyme preparation was preincubated as described under Materials and Methods with BSA and the cofactors and substrates indicated.

examined. Preincubating the enzyme preparation for 30 min in the presence of a concentration of ethylene similar to that generated in the reaction medium did not inactivate the enzyme (Table 2). Subsequently, the enzyme preparation was preincubated for 30 min in the presence of each cofactor and substrate, and the residual activities were determined (Figure 4). The enzyme was stabilized by preincubation in the presence of ACC, Fe^{2+}, and CO_2 (alone or in combinations) since the activities were in all cases higher than the activity of the enzyme preincubated without additions. The stabilization was maximum when the enzyme was preincubated with ACC + Fe^{2+} + CO_2 (92% of activity with respect to the control without preincubation). On the other hand, the enzyme was inactivated in the presence of ascorbate (alone or in combination with the other components of the reaction mixture), since the activities were always lower than the control preincubated without additions. The inactivation was maximum when the enzyme was preincubated with ascorbate + ACC + Fe^{2+} (17% of activity). The inclusion of CO_2 did not affect the inactivation (23% of activity), despite the absolute requirement of it on the catalytic turnover of the enzyme. Since pear enzyme has an absolute requirement of CO_2 for its activity, we conclude that complete reaction is not required for the inactivation and suggest that inactivation is the consequence of an enzyme−ACC−Fe^{2+}−ascorbate complex.

Exogenous Cofactors on in Vivo ACC Oxidase Activity. In vivo ACC oxidase activity is determined by adding plant tissues with exogenous ACC (Yang and Hoffman, 1984). In our experiments the pattern of extractable ACC oxidase activity was similar to that of in vivo activity. However, when the activities were expressed on a gram of fresh weight basis, higher activities were measured in vitro because they were carried out under optimal conditions. This indicated that in vivo ACC oxidase activity was probably being underestimated. Thus, we evaluated the in vivo ACC oxidase activity of pear tissues considering the requirements for the in vitro enzyme. Besides the substrate ACC, also Fe^{2+}, ascorbate, and CO_2 at the concentrations that optimized the in vitro assay were added to the in vivo reaction medium (Figure 5). The results

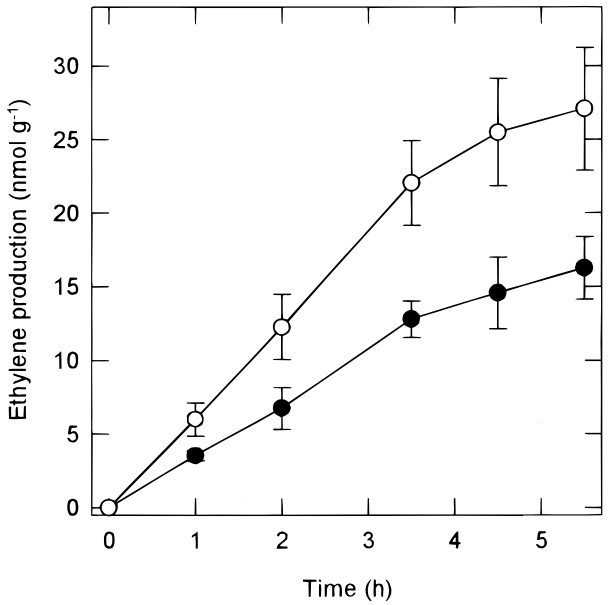

Figure 5. Time course of in vivo pear ACC oxidase activity in the absence (●) and presence (○) of exogenous cofactors (Fe^{2+}, ascorbate, and CO_2).

Table 3. Effect of Exogenous Cofactors on the in Vivo ACC Oxidase Activity Measured in Pear Disks[a]

	in vivo ACC oxidase activity	
added cofactors	nmol of C_2H_4 g^{-1} h^{-1}	%
none	2.88 ± 0.72	100
Fe^{2+}	5.20 ± 1.33	181
ascorbate	2.56 ± 0.29	89
CO_2	3.62 ± 0.36	126
Fe^{2+} + ascorbate	2.65 ± 0.68	92
Fe^{2+} + CO_2	9.49 ± 1.76	330
ascorbate + CO_2	3.76 ± 0.50	131
Fe^{2+} + ascorbate + CO_2	5.81 ± 1.04	202

[a] ACC oxidase activity was assayed after 2 h of incubation.

show that under these conditions the enzyme activity is higher than that resulting from incubating the tissues on ACC alone. This difference clearly indicates that in vivo ACC oxidase activity has not been assayed under optimal conditions. Figure 5 also shows that the ethylene production rate decreases during the time course, both in the presence and in the absence of exogenous cofactors, suggesting that an inactivation similar to that ocurring in vitro can be taking place in vivo.

The effect of the addition of each required cofactor and substrate on ACC oxidase activity in vivo was also studied (Table 3). ACC oxidase activity measured in pear disks was stimulated by the addition of 0.2 mM Fe^{2+} (181%) and 16% CO_2 (126%) and was inhibited by the addition of 10 mM ascorbate (89%). When the assay was carried out in the presence of ascorbate, Fe^{2+}, and CO_2, the activity was 2-fold (202%) that obtained in the absence of exogenous cofactors. Nevertheless, the greatest stimulation was achieved in the presence of Fe^{2+} and CO_2 (330%), showing again the inhibitory effect of ascorbate in vivo. The enzymatic nature of the stimulation by exogenous cofactors on ethylene production was corroborated with suitable controls. However, since the assay has been carried out at the concentration that optimized the in vitro assay and the cofactors and substrates have to be transported to the active site of the enzyme, their concentrations must still be optimized for the in vivo assay.

DISCUSSION

ACC oxidase is a peculiar member of the 2-oxoacid-dependent dioxygenase family. Ascorbate, which can be considered a cyclic 2-oxoacid (De Carolis and De Luca, 1994), is not a simple cofactor whose presence stimulates the in vitro activity, but it appears as an essential cosubstrate nonreplaceable by other reductants (Table 1). Thus, the inhibition of ACC oxidase activity by 2-oxoglutarate (Figure 1) would be the result of competition of this compound by the ascorbate-binding site of the enzyme, as reported by Iturriagagoitia-Bueno et al. (1996).

We have previously reported that pear ACC oxidase has a nonlinear time course in vitro (Vioque and Castellano, 1994a). This nonlinearity of the activity is due to inactivation during the catalysis. We found that ethylene did not inactivate the enzyme (Table 2). Besides ethylene, the other products of the reaction are dehydroascorbic acid and cyanide (Dong et al., 1992). Smith et al. (1994) and Mizutani et al. (1995) have reported that inhibition by dehydroascorbic acid or cyanide is unlikely to cause the nonlinear time course and inactivation of ACC oxidase, because both compounds are inhibitory only at high concentrations. Moreover, HCN is effectively metabolized into β-cyanoalanine in plant tissues (Yip and Yang, 1988). All of these data considered together permit us to reject end-product inhibition as the cause of the inactivation of ACC oxidase.

Nevertheless, we have demonstrated that ascorbate is responsible for the inactivation of pear ACC oxidase, which is maximum in the presence of ACC, Fe^{2+}, and ascorbate and that CO_2 is not involved in the inactivation, despite the absolute requirement of this cofactor for the enzyme activity (Figure 4). These results suggest that in pear the inactivation is due to the formation of a catalytically inactive enzyme−ACC−Fe^{2+}−ascorbate complex. Smith et al. (1994) suggested that the inactivation of tomato ACC oxidase is the consequence of an intermediate generated during catalytic turnover. These findings are in agreement with recent studies which have reported that the binding sites for ACC, Fe^{2+}, and ascorbate are intimate, whereas CO_2 binds to the enzyme at a different and distant site (Zhang et al., 1995).

With most 2-oxoacid-dependent dioxygenases, addition of catalase to the reaction medium at the relatively high concentrations of ~1 mg mL^{-1} optimizes the substrate turnover, especially during the later stages of purification (Prescott and John, 1996). A similar stabilization by catalase has been reported for several purified ACC oxidases (Smith et al., 1994; Zhang et al., 1995; Escribano et al., 1996), whereas crude extracts from melon did not show any protective effect of catalase (Smith et al., 1992). In contrast, we found an inhibitory effect of catalase on pear ACC oxidase activity (Figure 2).

The in vivo conversion of ACC into ethylene involves a stepwise cleavage of the cyclopropane ring of ACC (Adlington et al., 1982; Baldwin et al., 1988). Although very few in vitro mechanistic or structural studies have been reported, it is thought that the reaction proceeds via free radical intermediates (Acosta et al., 1993). As do other hydroperoxidases, catalase shows a dual function. In addition to decomposing H_2O_2, it is able to express a "peroxidative-like" activity (Ahmad, 1995). In this context, the inhibition of pear ACC oxidase activity

by catalase could be explained by its ability to react with hydroperoxides or free radicals generated during the catalysis that are required for the progress of the reaction. This suggestion is consistent with data reported by Vioque and Fernández-Maculet (1990), who showed that in vitro in the presence of molecular oxygen and a transition metal ion, peroxidase uses ACC as a substrate. Recently, Barlow et al. (1997) have proposed a catalytic scheme for ACC oxidase reaction that included an iron-linked peroxide intermediate.

Propyl-4-hydroxylase, a 2-oxoglutarate-dependent dioxygenase, binds to 2-oxoglutarate in a bidentade fashion: center I binds to the carboxyl in C5, and center II couples to C1 and C2 atoms in a metal-coordinated position (Majamaa et al., 1984). Studies with structural analogues of 2-oxoglutarate indicate that ACC oxidase could likely have two similar binding sites and CO_2 could bind to both of them (Dupille and Zacarías, 1996b). Considering the molecular structure of 2-oxoglutarate, we suggest that during the enzymatic conversion of ACC into ethylene, CO_2 binds to center I while ascorbate binds to center II in an Fe^{2+}-coordinated fashion. This would explain the inhibition of ACC oxidase activity by 2-oxoglutarate and why this inhibition has been found competitive with respect to both ascorbate (Iturriagagoitia-Bueno et al., 1996) and CO_2 (Dupille and Zacarías, 1996b). Furthermore, it provides a possible mechanism for the enzyme inactivation. If a portion of the ascorbate molecule resembles CO_2, ascorbate would also be able to interact with the CO_2-binding site of the enzyme, blocking the cofactor action and resulting in a loss of enzyme activity.

Our present data with pear tissues indicate that in vivo ACC oxidase is equally inactivated during the catalysis and that higher activities are obtained in the presence of exogenous cofactors (Figure 5). However, we also show that enzyme activity is inhibited by the addition of ascorbate (Table 3), despite the clear requirement demonstrable in vitro. Moya-León and John (1994) have previously found that ascorbate inhibited the in vivo ACC oxidase activity in banana fruits. They have also reported that the addition of Fe^{2+} and CO_2 stimulated the activity in pulp but not in peel tissues and that this stimulatory effect was dependent on the ripening stage.

In vitro ACC oxidase is inhibited at high concentrations of ascorbate (Vioque and Castellano, 1994b), the range of inhibitory concentrations being dependent on the CO_2 level (Finlayson and Reid, 1994; Mizutani et al., 1995). On the other hand, other 2-oxoacid-dependent dioxygenases are active in vivo in the absence of ascorbate (Prescott and John, 1996). Thus, in pear tissues ACC oxidase might utilize other different reductants with which the exogenous ascorbate would compete. Since the concentrations of cofactors used in these assays are higher than those likely to be required in vivo, it is premature to speculate on the nature of this inhibition.

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